Cellular identity of cerebellar memory

Izumi Fukunaga

University College London

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I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

The cerebellum is essential for some forms of motor learning and storage of motor memories. The simple organization of the cerebellum provides special opportunities to analyse mechanisms underlying learning and memory in a real neural network. A dominant hypothesis of cerebellar function is one based on suggestions by Marr and Albus, where Purkinje cell responses to parallel fibre inputs are modified under climbing fibre control. A candidate cellular mechanism is a long-term depression of parallel fibre-Purkinje cell synapses (pf-PC LTD) seen, in vitro, after conjunctive activation of parallel and climbing fibres. The associative and persistent nature of pf-PC LTD suggests that it may underlie behavioural forms of cerebellum-dependent learning.

Activation of the metabotropic glutamate receptor type 1 ($mGlu_1$ receptor) is critical for the induction of pf-PC LTD. If pf-PC LTD underlies learning, then blocking this receptor should lead to impairment of cerebellum-dependent learning. The studies described here test the role of the $mGlu_1$ receptor in one form of cerebellum-dependent associative learning.

Chapters 2 and 3 describe slice electrophysiological experiments that assess the mGlu1 antagonists CPCCOEt, YM-298198 and JNJ16259685. CPCCOEt was found to have a non-specific action, while YM-298198 and JNJ16259685 were found to be very potent and highly specific. Chapter 4 describes the effects of cerebellar infusions of JNJ16259685 on classical conditioning of the rabbit nictitating membrane response and reveals that conditioning was not impaired.

The result suggests there could be dissociation between pf-PC LTD and cerebellar learning. It is known that pf-PC LTD in vitro may not be a single phenomenon since it can be induced with a range of protocols that can differ significantly from the normal physiology. Thus, there may be a form of pf-PC LTD in vivo that is relatively independent of $mGlu_1$ receptor function. Alternatively, behavioural learning may depend upon plasticities involving other neuronal types in the cerebellar cortex.

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Abbreviations

aCSF	artificial cerebrospinal fluid
AIDA	(RS)-1-aminoindan-1,5-dicarboxylic acid
AIP	anterior interpositus nucleus
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BK	large conductance calcium-activated potassium channels
cf	climbing fibre
CGP66845	(2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPCCOEt	ethyl 7-(hydroxyimino)cyclopropa[b]chromen-la-carboxylate
CR	conditioned response
CS	conditional stimulus
DCN	deep cerebellar nuclei
DHPG	S-3,5-Dihydroxyphenylglycine
EPSP	excitatory postsynaptic potential
GABA	gamma-butyric acid
HVI	hemispheral lobule VI
LRN	lateral reticular nucleus
MCPG	(S)-α-methyl-4-carboxyphenylglycine
mf	mossy fibre
mGlu	metabotropic glutamate
$mGlu_1$ EPSP	$mGlu_1$-mediated excitatory postsynaptic potential
NBQX	2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NM	nictitating membrane
NMDA	N-methyl-D-aspartic acid
PC	Purkinje cell
pf	parallel fibre
pf-PC LTD	Long-term depression of parallel fibre-Purkinje cell synapses
PTD	post tetanic depression
rDAO	rostral dorsal accessory olive
SK	Small conductance Ca2+-activated potassium channels
UR	unconditioned response
US	unconditional stimulus
YM-298198	6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide hydrochloride

General introduction

1.1. The role of the cerebellum

It has been known for a long time that the cerebellum is involved in controlling movements and many inferences about its functions have been made from clinical investigations of patients with cerebellar damage (Holmes, 1939). Damage to the cerebellum does not seem to cause a complete loss of movements – so it cannot exert complete control over all aspects of movement. Instead, its role in control of movement must be more subtle. As described by Holmes (Holmes, 1939), the three fundamental signs of cerebellar damage are: (1) ipsilateral loss of muscle tone (hypotonia), (2) muscle fatigue (asthenia) and (3) loss of coordination, or ataxia, during voluntary or reflex movements. Loss of tone may be described as a decrease in the resistance to changes in muscle length. Loss of coordination after cerebellar damage may be seen during volitional movements as (i) dysmetria - the inability to make movements of precise amplitude due to alterations in rates and timings of muscle contractions and (ii) ataxia – the decomposition of movement where, for example, movements that are usually multi-joint are achieved via separate movements of individual joints, often leading to movement paths that are quite different from those of normal subjects. This loss of coordination can also occur in the control of posture, resulting in ataxia of gait. Overall, the cerebellum seems to be critical for making movements smooth and properly coordinated.

Recently, it has been suggested that functions of the cerebellum may extend to include cognitive functions. The human cerebellum, compared to the cerebella of other animals possesses proportionally larger hemispheres, or neocerebellum. This

expansion of the cerebellar hemispheres correlates with the expansion of the neocortex, to which higher or cognitive functions are often attributed. This extensive neocerebellar development has led to the suggestion that the human cerebellum is important in cognitive processes. In analyzing this suggestion it is important to be sure that putative cognitive functions of the cerebellum are investigated independently of motor functions. A few studies have addressed this problem by studying language processing in humans, using PET imaging (Petersen *et al.*, 1989). Subjects in this study were tested with a verb generation task, where they were asked to generate verbs associated with objects presented to them. During verb generation following visual presentations of each object, there was an increase in blood flow to the right posterior cerebellum, indicating increased activity in this part of cerebellum. The functional importance of this finding was supported by clinical studies on patients with infarcts in the distribution of the right posterior inferior artery (Fiez *et al.*, 1992). Patients tended to generate incorrect, non-verb answers and their performance did not improve after repeated tests. However, the nature of these deficits is complex because the patients are capable of performing well in similar tasks, e.g. a verb selection task, where they choose a verb from a list given to them, rather than generating their own. This indicates that the verb-generation deficit is not simply related to imagining movements during the task (Gebhart *et al.*, 2002).

Clinical investigations can give clues to functions of the cerebellum. However, in most cases, the lesions are not restricted and compensatory mechanisms can occur. Both factors complicate behavioural investigations so clinical cases are often not ideal for precise investigation of cerebellar functions. But studies in animals can provide the necessary experimental rigour. In designing such experiments to probe cerebellar

functions and their underlying mechanisms, knowledge of the anatomical organization of cerebellum is essential.

1.2. Cerebellum: gross anatomy

Neurons in the cerebellum are arranged within inner and outer parts, making up the cerebellar nuclei and cerebellar cortex, respectively. The cortex forms the outer layer, while the nuclei are found deeper in the structure, and so they are sometimes named the *deep* cerebellar nuclei. Rostrocaudally, the cerebellum is divided into 3 major parts: the anterior lobe and posterior lobe that are divided by the primary fissure and the flocculo-nodular lobe. In all mammals, this major division of the cortex can be further subdivided into a total of 10 lobules (Larsell, 1970). These 10 lobules are easily recognised in the vermis and are designated by the Roman numerals I-X in the rostrocaudal order (see Figure 1.1). Hemispheral lobules, which are extensions of these ten vermian lobules into lateral cerebellum, are correspondingly termed HII to HX (Figure 1.1). Some other, older nomenclatures are still in use for some lobules and are indicated in the figure.

Neurons of the cerebellar nuclei are found deeper in the tissue, amidst the white matter, and they are grouped into three nuclei. Most medially are neurons of the fastigial nucleus and the most lateral group forms the dentate nucleus. Between these two is the interpositus nucleus, which is further divided into the globose and emboliform nuclei in humans and other primates. In other species, the globose and emboliform correspond to anterior and posterior divisions of the interpositus nucleus complex.

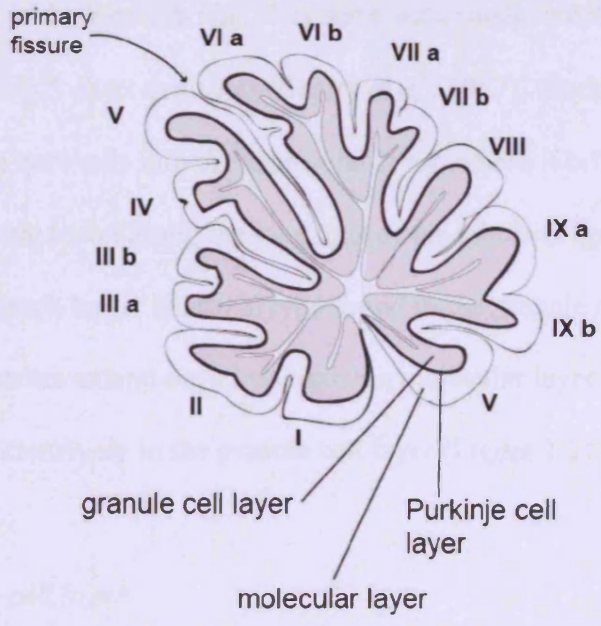


Figure 1.1. Gross anatomy and nomenclature of the cerebellar cortex.
Top illustration shows an external view of the cerebellum. The nomenclatures shown are used for the mammalian cerebellum (Bolk; left) and the human cerebellum (right). Larsell's nomenclature of the lobules, labelled I-X is shown on the left side of the vermis. Larsell's nomenclature is also shown in the parasagittal view of the mid vermis of a mammalian cerebellum (bottom figure). Three layers making up the cerebellar cortex are also illustrated. Top illustration from Voogd & Glickstein, 1998.

Although the external morphology of the cerebellum has these regional variations, the arrangement of neurons in the cerebellar cortex is remarkably regular. The neurons are arranged into three distinct layers: the granule cell, Purkinje cell and molecular layers (see Figure 1.1). The locations and morphology of these neurons are introduced in the section below.

1.2.1. Granule cell layer

The granule cell layer is the innermost layer of the cerebellar cortex, and as the name suggests, contains densely packed granule cells. Granule cells are the most numerous neuronal types in the whole brain. They have very small somata of about 5-8 μm diameter and with 5 short dendrites (Eccles *et al.*, 1967). Each granule cell sends an ascending axon outwards into the molecular layer, where it bifurcates to form two parallel fibres that travel along the long axis of the lobule (Figure 1.2). A second, less numerous but much larger neuronal type found in the granule cell layer is the Golgi cell. Their dendrites extend outwards, into the molecular layer and inwards, and their axons ramify extensively in the granule cell layer (Figure 1.2)

1.2.2. Purkinje cell layer

The Purkinje cell (PC) layer consists of the large cell bodies of Purkinje cells, arranged in a monolayer. Dendrites of Purkinje cells extend into the molecular layer where their extensive branches are covered with spines. Their dendritic trees are in a flat plane (Figure 1.2) perpendicular to the parallel fibres. PC axons are the sole output of the cerebellar cortex but they also collateralize to send projections within the cortex. Another neuronal type, with cell bodies close to the PC layer, is the Lugaro cell. Lugaro cell somata are less numerous and less regularly spaced than PCs and

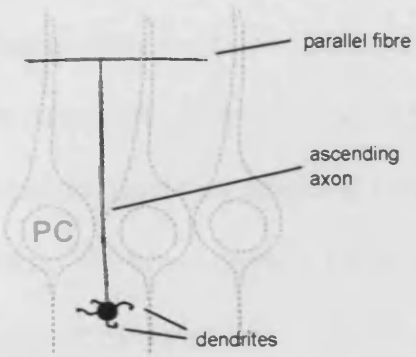
their dendrites and axons are known to course laterally and not extensively within the molecular layer.

1.2.3. Molecular layer

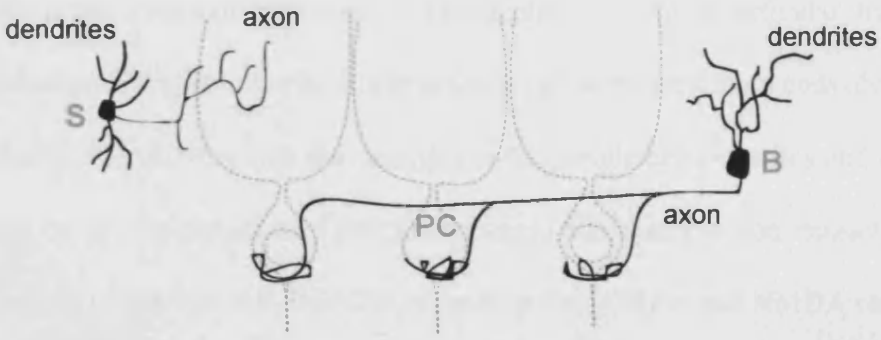
The molecular layer is packed with dendrites and axons but cell bodies are scarcer. Amongst the densely-packed parallel fibres and dendrites of Purkinje and Golgi cells, two types of interneurons, the stellate and basket cells, are distributed relatively sparsely. Stellate and basket cells are of similar size. Their dendrites and axons stay within the molecular layer and project (~1 mm in cats; Eccles et al., 67; ~0.3 mm in rats; Sultan and Bower, 1998) orthogonal to the flat plane of the PC dendrites (Figure 1.2). They are often classified together but they have distinct morphologies and different locations within the molecular layer. Stellate cells are found more superficially in the outer molecular layer whereas the somata of basket cells are found very close to Purkinje cell somata. The basket cells form a basket-like structure on Purkinje cells, near the axon hillock (Figure 1.2).

Figure 1.2. Morphology of cerebellar cortical neurons. (A) From a granule cell, the parallel fibre extends orthogonally to the flat plane of Purkinje cell dendritic trees (see below). (B) A Golgi cell; thick processes represent the dendrites, whereas thin processes represent the axonal ramification. The cells drawn in grey represent Purkinje cells viewed from the front. (C) A Purkinje cell perpendicular to (front) and in line with (side) of the course of parallel fibres (left) and flipped by 90° (right). (D) A Lugaro cell, whose processes run close to the Purkinje cell layer. (E) Stellate (S) and basket (B) cells; the cell body lies in the bottom 2/3 of the molecular layer. Its axon contacts Purkinje cells that lie transversely and wraps around the axon hillock of Purkinje cells. The Purkinje cells shown here are viewed from the front. (B) & (C) from Eccles et al., 1967; (D) from (Laine & Axelrad, 1996)

A


parallel fibre
ascending axon
PC
dendrites

E


dendrites
axon
S
dendrites
B
PC
axon

1.2.4. Deep cerebellar nuclei

The arrangement of neurons in the cerebellar nuclei is less uniform than in the cerebellar cortex. Three types of neurons are found in the nuclei and they can be distinguished by their size. The smallest neuronal type includes local inhibitory interneurons. The other two types define projection neurons. The largest neurons are glutamatergic and they project to various targets, including the red nucleus and the thalamus. The second type of projection neuron includes GABAergic neurons that project to the inferior olive. They are smaller than the glutamatergic projection neurons, but the soma sizes of the two populations overlap (Uusisaari *et al.*, 2007). Purkinje cells synapse upon both types of projection neurons in the cerebellar nuclei (de Zeeuw & Berrebi, 1995).

1.3. Flow of information in the cerebellum

1.3.1. Granule cell layer

There are two distinct classes of glutamatergic afferent fibres to the cerebellar cortex. The first class defines the mossy fibres that project to the granule cell layer. Mossy fibres come from a diverse range of brainstem (e.g. pontine, reticular, trigeminal, vestibular) and spinal nuclei. In the granule cell layer they form convoluted structures called glomeruli, in which they synapse with granule cell dendrites and in which Golgi cell axons terminate. Fast glutamatergic transmission from mossy fibre terminals to granule cell dendrites is mediated by AMPA and NMDA receptors. The arrangement of terminals and dendrites within the glomeruli causes glutamate released at one synapse to have significant influence on neighbouring synapses, producing transmission by glutamate spillover (DiGregorio *et al.*, 2002). Each granule

cell dendrite is thought to enter a different glomerulus, thereby potentially allowing the integration of different modalities within a granule cell but whether such an integration actually occurs is still debated (Chadderton *et al.*, 2004; Jorntell & Ekerot, 2006). The efficacy of mossy fibre inputs to the granule cells is influenced by the GABAergic inhibition from Golgi cells. This inhibition can occur in tonic and phasic modes that refer to action potential-independent and dependent release of GABA, respectively. Both forms of inhibition can undergo modulation. For example, the tonic release of GABA can be modulated by acetylcholine (Rossi *et al.*, 2003) and phasic release of GABA is influenced by glutamate release from mossy fibres (Mitchell & Silver, 2000).

1.3.2. Molecular layer

Granule cells project into the molecular layer to form synapses on the dendrites of Purkinje cells, Golgi cells, stellate and basket cells (Eccles *et al.,* 1967). Both ascending axons and parallel fibres make synapses on Purkinje cells, but the synapses from these different segments of the efferent axon are reported to be functionally different (Bower, 2002). Parallel fibres extend, depending on the species, a few millimetres and therefore potentially contact a large number of Purkinje cells. An anatomical observation suggests that each parallel fibre may contact about 50% of the Purkinje cells it encounters (Harvey & Napper, 1991). It has also been suggested that most (93%) of these synapses are silent (Isope & Barbour, 2002). However, because of the great number of parallel fibres, collectively they produce a powerful influence on Purkinje cells (Eccles 1973). Fast glutamatergic transmission to PCs is mediated by AMPA receptors, but glutamate also has a slow postsynaptic influence via $mGlu_1$ receptors. Parallel fibre inputs to Purkinje cells can also be modulated presynaptically

via activation of either CB_1 receptors (Levenes *et al.,* 1998; Takahashi & Linden, 2000; Brown et al., 2003) or $mGlu_4$ receptors (Lorez *et al.,* 2003).

The influence of parallel fibre activity on stellate and basket cells provides feed-forward inhibition in the molecular layer: excitation of stellate and basket cells by parallel fibres causes a subsequent inhibition of the target neurons, including Purkinje cells. Stimulation of bundles of parallel fibres reveal two types of feed-forward inhibition: on-beam and off-beam. On-beam inhibition of Purkinje cells is provided by stellate and basket cells that lie on the path of parallel fibres contacting the same Purkinje cells, and it has the effect of making parallel fibre excitation of Purkinje cells briefer (Eccles *et al.*, 1967; Mittmann *et al.*, 2005). Stellate and basket cells also provide "off-beam" inhibition of Purkinje cells where they can inhibit Purkinje cells that do not lie on the path of activated parallel fibres (Eccles *et al.*, 1967), because their axons extend transversely (Figure 1.2). However, studies using somatosensory stimulations suggest that the postsynaptic effect of parallel fibres on Purkinje cells may be different from that on interneurons that lie on the parallel fibre path (Ekerot & Jorntell, 2001; Jorntell & Ekerot, 2003). In addition, activation of Golgi cells by parallel fibres provides a feedback inhibition to mossy fibre-granule cell transmission (Eccles *et al.*, 1966c).

Climbing fibres form the second class of glutamatergic input to the cerebellar cortex. Their cell bodies are in the contralateral inferior olivary nucleus of the medulla and their major targets in the cerebellar cortex are Purkinje cells. Each fibre ramifies extensively in the molecular layer and makes multiple contacts with the target Purkinje cell (Figure 1.3) but each Purkinje cell is contacted by only one fibre from

one olivary neuron (Eccles *et al.*, 1966b). This arrangement leads to an exclusive yet powerful excitatory effect produced by the climbing fibre in each Purkinje cell. This response is known as the complex spike, and it consists of a large depolarisation, followed by a long lasting (~100 ms) plateau potential upon which rides a burst of spikelets (~500 Hz) during the first 10 ms (Llinas & Sugimori, 1980b; Schmolesky *et al.*, 2002). The most significant consequence of the climbing fibre response in Purkinje cells is the substantial rise in intracellular calcium concentration ($[Ca^{2+}]$) produced by activation of voltage-gated calcium channels (Konnerth et al., 1992).

Figure 1.3. The arborisation of a climbing fibre in the molecular layer. A single climbing fibre filled with and stained for biotinylated dextran amine was traced. The fibre ramifies extensively around the main dendrites of the Purkinje cell. The left and right figures show the climbing fibre ramifications viewed from the 'front' and 'side', respectively. From Wu *et al* (1999).

Climbing fibre responses have often been described as all-or-none in character. This is due to the presence of only one climbing fibre innervating a particular Purkinje cell, as well as a highly secure transmission at the climbing fibre to Purkinje cell synapse; activation of a climbing fibre always results in a powerful postsynaptic response in the Purkinje cell. Therefore, when stimulating a climbing fibre near its threshold of activation, the postsynaptic response to successful or unsuccessful activations of the climbing fibre appears all or none, respectively. This often conjures up an idea of climbing fibre responses whose waveform is invariant under all conditions. This may be true in the special case where the climbing fibre is stimulated at low frequency (up to ~1 Hz), in isolation from other synaptic events. However, climbing fibre synapses can undergo short- and long-term plasticities (Hansel & Linden, 2000; Konnerth *et al.*, 1990). The waveforms and cellular consequences of climbing fibre responses are affected by other synaptic inputs to the Purkinje cell. For example, the number of secondary spikelets during a climbing fibre response can be substantially reduced by inputs from molecular layer interneurons (Eccles *et al.*, 1966).

As we have seen, the inputs from parallel fibres, molecular layer interneurons and climbing fibres converge in Purkinje cells and influence their spiking patterns. These firing patterns are conveyed via GABAergic Purkinje cell axons to the deep cerebellar nuclei (DCN) or to the vestibular nuclei. The DCN also receive collaterals of glutamatergic mossy and climbing fibres, whose fast transmission is mediated by AMPA, as well as NMDA receptors (Gauck & Jaeger, 2003; Pugh & Raman, 2006). In DCN slice preparations, the projection neurons are reported to be spontaneously active so that they are able to generate action potentials without excitatory synaptic drive. However, *in vivo*, the DCN neurons are usually under a substantial amount of

GABAergic inhibition, due to ongoing Purkinje cell activities and a synchronous pause in their firing can produce an output of the DCN neurons (Gauck & Jaeger, 2003). It is not yet clear exactly which patterns of inputs to the DCN lead to the generation of action potentials in the projection neurons, and whether pauses in Purkinje cell axons or increases in mossy or climbing fibre collaterals alone are sufficient to drive behaviourally relevant outputs as has been suggested (Albus, 1971; Marr, 1969).

1.4. The Marr-Albus model of cerebellar cortex; a classical view of cerebellar function

How can the neuronal elements described above be "fitted" together to form a model information processing architecture? A dominant hypothesis incorporating the anatomical and physiological properties of the cerebellum arises from original and independent suggestions by Marr and Albus (Albus, 1971; Marr, 1969). Both suggested that the cerebellum is involved in adaptive control and the site of modification for the storage of memory is at the parallel fibre-Purkinje cell synapses. In both hypotheses, a subset of granule cells is activated by a pattern of mossy fibres, and the resulting combination of parallel fibre activities represents a sensory environment. Marr (1969) suggested that the connections between the parallel fibres and Purkinje cells are strengthened (Marr, 1969) and Albus (Albus, 1971) suggested they are weakened under climbing fibre control. In Marr's hypothesis, the result of such modification at the parallel fibre-Purkinje cell synapses is to increase the firing rate of Purkinje cells in response to a learnt pattern of pf inputs. Marr was aware of the inhibitory nature of Purkinje cells on their target, and suggested that there exists a sign converter somewhere in the output pathway in order to make this increased

inhibitory Purkinje cell output a driving force for a movement. Albus, on the other hand, suggested that the Purkinje cells respond to learnt parallel fibre patterns with pauses in the simple spikes. He likened the process of modifying the parallel fibre-Purkinje cell synapses under a climbing fibre control to classical conditioning. That is, a climbing fibre input to the Purkinje cell was likened to an unconditional stimulus, and a short pause in Purkinje cell simple spikes that follows a climbing fibre input an unconditioned response. The pause that the Purkinje cell generates in response to a learnt pattern of parallel fibre activity was likened to a conditioned response.

Both Marr and Albus suggested functions of the other cortical neuronal types. The other GABAergic neurons in the cerebellar cortex i.e. Golgi, stellate and basket cells, were assigned to regulating the overall amount of excitation present at any time in the cerebellar cortex. Feedback inhibition in the granule cell layer provided by the Golgi cells would maintain the proportion of active granule cells at a fixed level. The feedforward inhibition provided by the molecular layer interneurons would provide a normalising function so that, for example, excitation upon one branch of a Purkinje cell dendritic tree that receives a greater number of parallel fibres would not overshadow another branch that receives less parallel fibre inputs but which is of equal importance for Purkinje cell control. Albus further postulated that the parallel fibre inputs to stellate and basket cells are also modifiable, and thus memory storage capacity could be increased.

1.5. On the nature of signals carried by climbing fibres

In both Marr's and Albus' hypotheses, the climbing fibre plays a crucial role in 'instructing' the Purkinje cells in selecting the appropriate patterns of parallel fibre

synapses to be modified. Empirical evidence that suggests such a role of climbing fibre activity i.e. the activity signalling the error of intended movements, was later obtained from numerous single unit recordings from Purkinje cells, where conditions under which complex spikes occurred were investigated. For example, in one study, monkeys were trained to hold a handle within a restricted zone and return it quickly to the zone when disturbed by a sudden force perturbation of consistent magnitude. When the magnitude of the disturbance was changed the monkey needed a series of trials to learn the new force disturbance (Gilbert & Thach, 1977). In some Purkinje cells, complex spike occurrences were observed when the magnitude of the disturbance force was changed and, as the monkeys learned the task, the complex spike occurrence decreased.

Similarly, in awake, behaving cats, recordings from Purkinje cells in the lateral part of the vermis show that the occurrence of climbing fibre activation is only very weakly linked to a specific phase of the normal step cycle, but is strongly linked to an unexpected perturbation during walking. When the cats walked over a loose rung, that dropped when the foot stepped on it, complex spikes were reliably observed before the rung reached the bottom of travel (Andersson & Armstrong, 1987).

While the exact event that led to the complex spike occurrence in the above studies is difficult to determine, the climbing fibre events in Purkinje cells in the flocculus during vestibular stimulation gives a clearer indication of the signals carried by climbing fibres. Purkinje cells in the flocculus receive visually driven climbing fibre inputs (Maekawa & Simpson, 1972). The climbing fibres signals here are related to image motion on the retina, which is direction and speed specific. The image motion

is produced by an error in the eye movements in stabilising the image on the retina during head movements. Therefore, the climbing fibres may be said to carry the sensory consequence of the error in movements. And because such signals report whether a movement was successful or not, the idea of an instructive role of climbing fibres suggested by Marr and Albus is supported (Simpson *et al.*, 1996).

The nature of climbing fibre signals may, however, be more complex than suggested above. This complexity is illustrated by the following two studies. First is a study by Kitazawa *et al.*(1998), where monkeys were trained to reach to visual targets that appreared at random locations on a screen (Kitazawa *et al.*, 1998). Each target appeared when the monkey pushed a button to start a trial and vision of the target and of the hand was blanked during the reach and restored when the reach was complete. Complex spikes were seen early in the reach period and after reach completion. Although the late responses related to response error, the early responses were related to reach direction

In another study, complex spike occurrences were analysed in saccade-related Purkinje cells in the vermis of lobules V and VI (Catz *et al.*, 2000). Monkeys first fixated on the centre of a screen, and then made saccadic eye movements to a target that appeared at a wide range of positions in order to foveate on the target. In a saccadic adaptation task, the position of the target was shifted by a fixed amount during saccade. Initially, the saccade missed the displaced target so the monkeys had to make corrective eye movements. But the subjects adapted to the task by changing the amplitude of their saccadic movements. Contrary to an expectation that greatest occurrences of complex spikes might be found before the saccadic adaptation takes

place, when the errors are the greatest, Catz *et al.* (2000) observed that the complex spike occurrences gradually increased during learning and were maintained after the adaptation had taken place.

Despite these complexities of climbing fibre signalling, ideas of cerebellar functions based on the Marr-Albus hypotheses are still the most coherent and consistent with empirical evidence, four decades after their first description. The Marr-Albus idea introduced above is envisaged to apply uniformly for all regions of the cerebellum. However, is it reasonable that the variety of functions in which the cerebellum is involved can be explained by implementing this single algorithm? One hypothesis is that the cerebellum contains many modules i.e. Purkinje cells in the cerebellum can be grouped into small units according the inputs they receive and the structures which they target. This would give the Purkinje cells within each unit a common function, for example, to influence a particular motor pool. Therefore a module can be said to be a functional unit. In this way, the same algorithm could be used to achieve different cerebellar functions. How are such modules organised?

1.6. Regional specificity – zonal structures and microcomplexes defined by climbing fibre inputs

The modular organisation of the cerebellum is defined by the projection patterns of climbing fibres (Andersson & Oscarsson, 1978). Climbing fibres from different compartments within the inferior olive project to the cerebellar cortex in a series of parasagittal strips. This parasagittal organisation is illustrated in the Figure 1.4. Zones can be distinguished electrophysiologically by climbing fibre responses to peripheral stimulations. Zones can be defined by their receptive field properties: the stimulus

quality, laterality and location of stimulation that produces a response and the response latency. A microzone is functionally distinguished as a strip of Purkinje cells within which the climbing fibres signals have the same property (Apps & Garwicz, 2005).

This specific climbing fibre projection pattern is preserved in that the Purkinje cell projection to the DCN has a specific pattern, matching those of corresponding climbing fibre collaterals. In other words, those climbing fibres projecting to a set of Purkinje cells have collateral projections to the same region of DCN that receives input from those Purkinje cells (Figure 1.4). This forms a functional unit, or module, termed the cerebellar microcomplex (reviewed in Apps & Garwicz, 2005).

Figure 1.4. Climbing fibre projection patterns in the cerebellar cortex and cerebellar and vestibular nuclei of the rat. Regions of the inferior olive, and the corresponding regions to which climbing fibres and collaterals project to are pattern-coded. From Voogd & Ruigrok (2004)

1.7. The mossy fibre projection pattern

Mossy fibres also have discreet projection patterns. Patches of the granule cell layer represent a fragmented map of body parts defined by the signal carried by mossy fibres projecting to them (Shambes *et al.*, 1978). It has also been suggested that mossy fibres projecting to a patch of granule cells respond to stimuli similar to those that excite the climbing fibres innervating the overlying Purkinje cells (Garwicz *et al.*, 1998). However, this correspondence between the climbing fibre and mossy fibre projection patterns is not strict and the degree of similarity may differ from one climbing fibre microzone to another (Garwicz *et al.*, 1998). Could different mossy fibre afferent systems have different degrees of correspondence between their termination patterns and those of the relevant climbing fibres? That is, are the mossy fibre projection patterns less similar to the projection pattern of climbing fibres for mossy fibres systems that project more widely? For example, mossy fibres whose cell bodies lie in the trigeminal and cuneate nuclei have more restricted projection patterns than those from the LRN (Wu et al., 1999, Ji & Hawkes 1994, Sultan, 2001). However, even in the case of trigeminal and cuneate systems, their mossy fibre terminals are found bilaterally and in bands that are scattered underneath six zebrin bands (Ji & Hawkes 1994).

In principle, therefore, cerebellar modules could be defined by their mossy fibre projection patterns. Since the sole output of the cerebellar cortex is by Purkinje cell axons, the unit of function is determined by Purkinje cells. Thus, the relationship between the activities of local mossy fibres and Purkinje cells is critical. However, if the mossy fibre postsynaptic influence is largely determined by the actions of the

parallel fibres, instead of the ascending axons, then any topography present in the granule cell layer is likely to be lost by the long parallel fibres.

1.8. Ascending axon vs parallel fibres: which one influences the Purkinje cell activities more strongly?

There have been suggestions that the granule cells ascending axonal synapses are the dominant determinant of Purkinje cell function (Bower, 2002). It is true that the activation of individual ascending axon produces a stronger postsynaptic response than individual parallel fibre synapses (Isope & Barbour, 2002). However, given their vast number, the parallel fibre inputs, collectively, are able to produce significant postsynaptic response in Purkinje cells. An answer to the relative importance of ascending axons and parallel fibres in defining Purkinje cell functions may be found by analysing the firing patterns of Purkinje cells *in vivo* with respect to the local or distant granule cells i.e., whether the Purkinje cell firing patterns match those of the local granule cells. Local granule cells are likely to contact the Purkinje cells via ascending axons, while distant granule cells that are likely to contact Purkinje cells via parallel fibres. Thus, a close resemblance between the firing patterns of Purkinje cells and local granule cells may support the significant role of local granule cells in defining the Purkinje cell functions. Bower and Woolston (Bower & Woolston, 1983) observed that the Purkinje cells responded to the same stimuli as the underlying units in the granule cell layer. However, there are contradictory findings, for example, receptive fields of Purkinje cell simple spikes, i.e. stimuli that increase the frequency of Purkinje cell simple spikes, did not correspond to the receptive fields of local climbing fibres and mossy fibres lying underneath the recorded Purkinje cell (Ekerot & Jorntell, 2001).

As we have seen, the modular structure of the cerebellum is currently more clearly defined by its climbing fibre inputs than by its mossy fibres inputs. So, in the studies to be described in this thesis, any regional specification is defined by the former. Modular organisation of the cerebellum in the form of physically distinct microcomplexes implies that complex actions involve many microzones of Purkinje cells, possibly scattered over a large area of the cerebellum. In other words, for rigorous investigations of cerebellar functions, especially when local manipulations of relevant cerebellar regions are required, we need a simple behaviour that involves as few modules as possible. One example of a well-characterised, simple behaviour that requires normal cerebellar function is classical conditioning of the nictitating membrane response in the rabbit. This learning behaviour is further analyzed in the work described in this thesis.

1.9. A simple, cerebellum-dependent behaviour: rabbit nictitating membrane response conditioning

One of the best-characterised, simple cerebellum-dependent behaviours is classical conditioning of the nictitating membrane (NM) response in the rabbit. The NM is also called the third eyelid, and is a protective membrane that passively moves across the cornea upon retraction of the eyeball by the retractor bulbi muscle (Figure 1.5). This muscle is controlled by neurons in the accessory abducens nucleus (Gray *et al.*, 1981). There is no antagonist muscle and the eyeball and NM return to a resting position due to elastic properties of the tissues. Thus, the behaviour is controlled by a single muscle. This is in contrast to the movement of the upper external eyelid, which is

controlled by a pair of antagonistic muscles, namely, the orbicularis oculi and levator palpebrae (Figure 1.5) that lower and raise the upper eyelid, respectively.

Figure 1.5. **The mechanisms of NM and external eyelid closure.** A cross section (left) shows an eyeball viewed from the side and 3 types of muscles involved in the movement of NM and external eyelid. Retractor bulbi muscles (2 out of 4 are shown) are attached to the back of the eyeball, near the exit point of the optic nerve. A contraction of these muscles results in the retraction of the eyeball, which results in a passive movement of the nictitating membrane over the cornea (right). A contraction of the ring shaped orbicularis oculi muscle results in the closure of the external eyelids, whereas a contraction of levator palpebrae opens the eyelids. The black arrows indicate the directions of muscle contraction. Abbreviations: r.b. retractor bulbi; l.p. leavator palpabrae; o.oc. orbicularis oculi. Based on Yeo & Hesslow, 2002

Normally, closure of the NM is reflexive to certain stimuli such as periocular stimulation and, as the closure of the NM is not under voluntary control, spontaneous responses are minimal. During NM response conditioning, a previously neutral stimulus, or conditional stimulus (CS), is repeatedly presented together with a stimulus that elicits the reflex, the unconditional stimulus (US). Examples of CS types include tone, light and touch, and examples of US types include corneal air puff and

periorbital electrical stimulation. Ultimately, after a sufficient number of paired CS and US trials, the CS itself is able to drive the NM response. This learnt closure of the NM in response to the CS is called a conditioned response (CR). The peak amplitude of such responses is well timed to the CS-US interval used. At the asymptote of conditioning, the conditioned responses are observed almost every time a CS is presented (i.e. %CR = 100). Furthermore, the acquisition, as well as execution of conditioned NM response requires the normal cerebellar function. The critical cerebellar regions involved in the NM response conditioning are described below.

1.10. Cerebellar cortical regions critical for NM response conditioning

Physical lesions of the cerebellar cortex have devastating effects on acquisition and execution of the learnt NM response (Yeo *et al.*, 1985b). Lesions including the ipsilateral hemispheral lobule HVI lead to the loss of conditioned responses as well as impairments in their acquisition. Tracing studies show that C1 and C3 climbing fibre zones of lobule HVI receive climbing fibres from the medial part of rostral dorsal accessory olive (DAO; Yeo *et al.*, 1985, Sugihara & Shinoda, 2004). This region is known to relay somatosensory information from the face (van Ham & Yeo, 1992). Mossy fibres projecting to this region include those whose cell bodies lie in the basilar pontine nuclei, which receive projections from the inferior colliculus (Yeo *et al.*, 1985c). Thus, with periocular stimulation US and an auditory tone CS, lobule HVI receives US and CS related information via climbing fibres and mossy fibres, respectively.

Consistent with the lesion and projection studies, local reversible inactivation using drugs show that ipsilateral HVI is required for normal acquisition, as well as

execution of the conditioned responses. For example, local infusion of the AMPA/kainate receptor antagonist CNQX during acquisition substantially impairs the conditioning (Attwell *et al.*, 2001). CNQX infusion here also blocks the expression of learnt NM responses (Attwell *et al.*, 1999).

Which cerebellar module is involved in the rabbit NMR conditioning? Electrophysiological studies indicate that the C1 and C3 zones in HVI and HVII receive short latency cf response evoked by the unconditional stimulus (electrical stimulations of ipsilateral periorbital region; Figure 1.6; Hesslow, 1994). In particular, an analysis of the spread of tritiated CNQX that caused effective block of CR execution suggested that the C3 zone in HVI, corresponding to a region between Zebrin P4b+ and P5+ strips (Figure 1.6) is critical for NM response conditioning (Attwell *et al.*, 1999; Sanchez *et al.*, 2002). A further electrophysiological mapping study has suggested an existence of another blink-related patch in the deeper region of HVI, namely in the zebrin P5+zone in some cases (Millar, PhD thesis, University of London, 2002). No short-latency climbing fibre responses to ipsilateral periocular stimulations have been found in the lateral wall of HVI (L. Millar, PhD thesis, University of London, 2002).

Purkinje cells from this zone project to the anterior interpositus nucleus that has a prominent output to the red nucleus. A population of neurons in the red nucleus is known to project to the accessory abducens nucleus, which in turn project to the retractor bulbi muscles (Gray *et al.*, 1981). This circuitry is illustrated in Figure 1.7. The evidence supports the suggestion that HVI is a site for convergence of auditory mossy fibre information related to the CS (Kandler & Herbert, 1991, Kawamura 1975,

Yeo *et al.,* 1985c) and climbing fibre signals related to the US, and that the motor pools it controls include that of the retractor bulbi muscle, which drives the NM response.

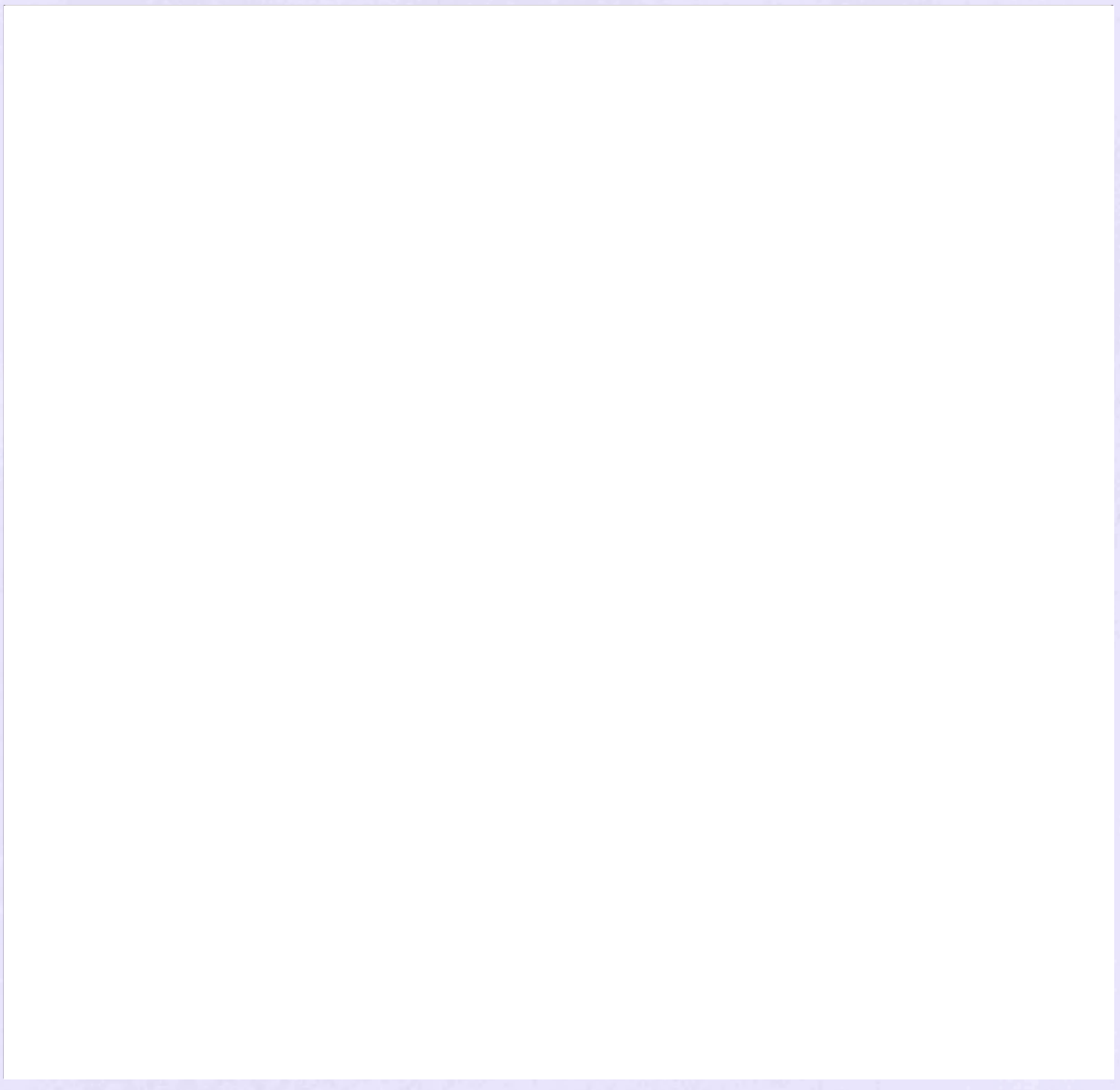


Figure 1.5. A climbing fibre zone critically involved in the NM conditioning. (A) A zebrin (anti-aldolaseC)-stained rabbit cerebellum viewed from front. The immune-positive bands are medio-laterally numbered. P5+ and P6+ are the 5th and 6th Zebrin positive bands from the mid line.The location of lobule HVI is highlighted with a rectangle. (B) A transverse section of zebrin-stained lobule HVI. Area important for expression of CRs is indicated in blue and is found between the 5th and 6th zebrin positive (P5+ and P6+) bands. (C) An external view of a cat cerebellum. Dark parasaggital bands indicate regions where Purkinje cells receive short latency climbing fibre response to ipsilateral periocular stimulations. (A) from Sanchez et al., 2002, (B) from Attwell et al., 1999, (C) from Hesslow, 1994.

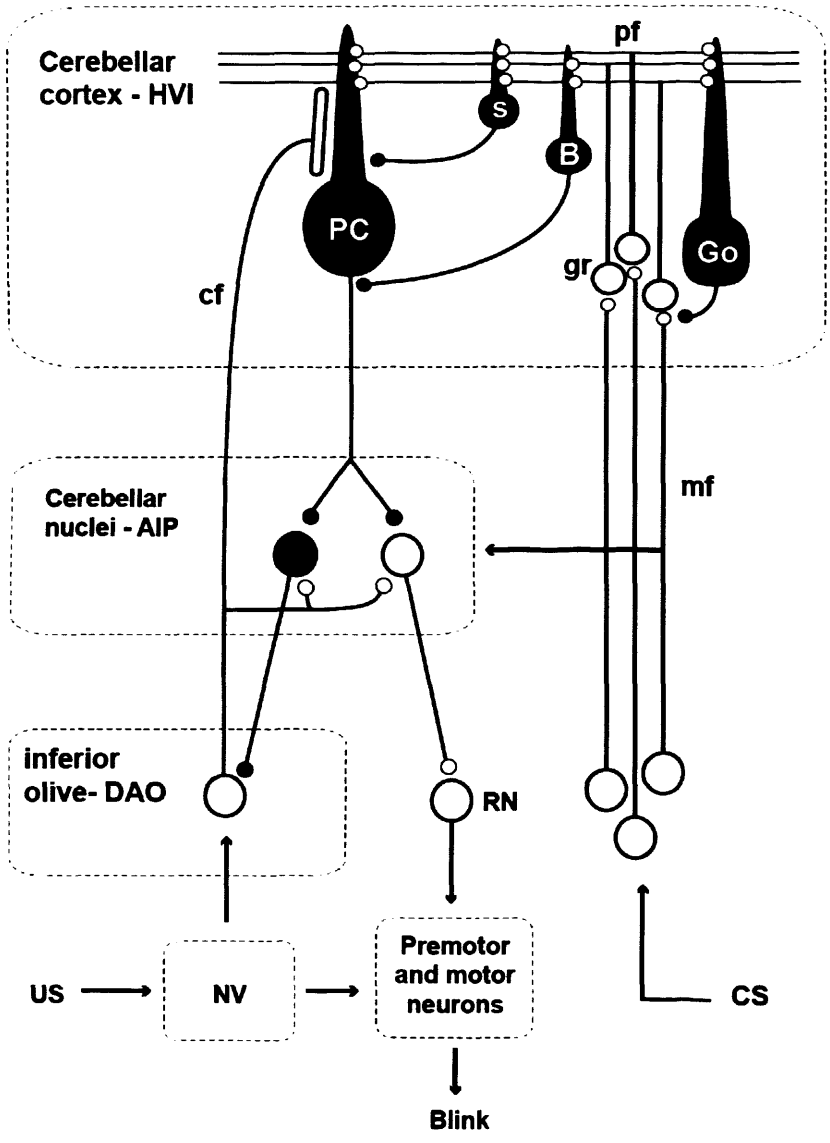


Figure 1.6. Circuitry involved in NM response conditioning. US-UR pathway is relayed via the trigeminal nucleus (NV) to the premotor and motor neurons in the accessory abducens, facial and oculomotor neuclei, that control the blink reflex. The US- and CS-related signals converge on Purkinje cells in the C1/C3 zone of the lobule HVI via climbing fibres and mossy fibre-granule cell axons, respectively. Climbing and mossy fibres also send collaterals to the AIP. The output of this microzone converges on the premotor and motor neurons controlling the blink via several intermediate structures, including the anterior interpositus nucleus and the red nucleus. Glutamatergic neurons are depicted by large open circles, whereas GABAergic neurons are depicted by large filled circles. Arrows indicate connections whose details are unknown or unspecified. Abbreviations; AIP anterior interpositus nucleus, B basket cell, cf climbing fibre, CS conditional stimulus, DAO dorsal accessory olive, Go Golgi cell, gr granule cells, mf mossy fibres, NV trigeminal nucleus, PC Purkinje cell, RN red nucleus.

1.11. Involvement of anterior interpositus nucleus in eyeblink conditioning

The anterior interpositus nucleus (AIP) is known to be crucial for the acquisition, as well as the execution of the conditioned NM response, since lesion of the AIP leads to loss of NM response conditioning (McCormick & Thompson, 1984; Yeo *et al.*, 1985a). Also, local infusion of the $GABA_A$ receptor agonist, muscimol, in the AIP during conditioning has a devastating effect on acquisition of NM response conditioning and, in conditioned subjects, impairs or abolishes the execution of learnt responses (Krupa and Thompson 1993; Hardiman *et al.*, 1996). Since AIP is a target of Purkinje cells in HVI, as well as of climbing fibre collaterals originating from the rostral dorsal accessory olive, there is convergence of CS- and US-related signals in the DCN. Furthermore, the disruption of memory formation with pharmacological inactivation of the cerebellar cortex could also be explained in terms of nuclear plasticity, by preventing transmission of CS and US-related signals to the DCN. This problem of distinguishing the effect of the drug on block of signal transmission from that of where the memory is stored has been answered to some extent by a study investigating the memory consolidation. An infusion of muscimol in the AIP after acquisition training sessions does not lead to impairment in the consolidation of learning, whereas a similar treatment of the HVI does (Attwell *et al.*, 2002). This indicates that a mechanism seems to operate in the cortex, rather than the DCN, in a period after a conditioning, which is crucial in laying down the memory. As it will be explained in chapter 4, the proposed behavioural experiments in this thesis may also assist in dissociating sites of memory formation.

1.12. Summary

The way in which the cerebellum controls movements is closely related to its modular structure. Cerebellar modules are functional units defined by their climbing fibre inputs. The region of cerebellum critically involved in one simple behaviour, the classically conditioned NM response, is a C3 climbing fibre zone in lobule HVI ipsilateral to the movement. In seeking to understand how the cerebellum controls this simple behaviour, we can draw upon numerous models. A dominant view in many useful models is that an association of context for movement occurs at pf-PC synapses under climbing fibre control, as first suggested by Marr and Albus. Can we now start to address whether the functions contained within the Marr-Albus hypotheses really are implemented in the cerebellum?

1.13. Long-term depression of the parallel fibre-Purkinje cell synapses: a possible cellular mechanism for the implementation of the Marr-Albus model?

Ito and colleagues first described a form of synaptic plasticity consistent with an implementation of the Marr-Albus hypotheses (Ito *et al.*, 1982; Ito & Kano, 1982). This plasticity is a long-term depression of the efficacy of synaptic transmission from parallel fibres to Purkinje cells (pf-PC LTD). This pf-PC LTD can be induced by repeated conjunctive activations of climbing fibre and parallel fibres (sometimes activated via mossy fibres). In this early work, Ito and colleagues explored whether this form of plasticity mechanism might play a role in the adaptation of gain in the vestibulo-ocular reflex, a form of learning that depends upon the cerebellar cortical flocculus (reviewed in Ito, 1982). This idea was consistent with the nature of the signals arriving at the flocculus. Mossy/parallel fibres carry vestibular information

relating to rotational head movement, and the climbing fibre signals carrying a retinal slip signal as the sensory consequence of the error in the compensatory eye movement.

1.14. Pf-PC LTD – a candidate mechanism for NMR conditioning?

Could pf-PC LTD be an important mechanism that underlies classical conditioning of the rabbit NM response? A first step in answering this question is to determine whether NM response conditioning and pf-PC LTD share similar properties.

1.14.1. Associativity

As with other forms of associative learning, eyeblink conditioning involves the association of two stimuli, the CS and US. When the CS and US have a random temporal relationship to each other, conditioned responses do not develop. Successful classical conditioning requires contingency and contiguity (Gormezano *et al.*, 1983). Therefore, any candidate cellular mechanism should also have these requirements. Originally, pf-PC LTD was thought to be induced only after conjunctive activations of pf and cf. With the size of pf stimulation used in these experiments, parallel fibre stimulation alone produces a long term potentiation of the pf-PC synapse (Sakurai 1987; Coesmann et al., 2004). It is this property of pf-PC LTD that gives it credibility as a mechanism for this and other forms of cerebellum-dependent behavioural learning. As will be discussed later, pf-PC LTD can have different induction mechanisms depending upon the protocol used, some of which are not necessarily associative. For example, pf-PC LTD can be induced by pf stimulation alone, if the pf stimulation is sufficiently strong. It is important to question how physiologically relevant the "strong" activation of parallel fibres is in such protocols. However, parameters that determine the strength of parallel fibre inputs to Purkinje cells during

CS presentation, such as the number and the density of active granule cell axons, are currently unknown and therefore it is difficult to comment on the plausibility and physiological relevance of any particular stimulation protocol.

Here, we focus on a version of pf-PC LTD whose induction is known to be associative and to require repeated pairings. In these two properties, it closely matches our target behaviour. NM response conditioning is not a one-trial learning – it requires repeated presentations of paired CS and US and it is essentially associative.

1.14.2. Temporal contiguity

Another factor that is common both to pf-PC LTD induction and to NM response conditioning is that not all patterns of pf/cf stimulation and CS-US presentations lead to induction of plasticity or conditioning respectively. In both cases, the rate of induction depends on the interval between the two stimuli. The effect of varying the interval between CS and US (CS-US interval) on the rate of conditioning can be seen as a bell-shaped curve; when the CS-US interval is very short (<100 ms) or very long (>1000 ms), it takes very much longer to reach an asymptotic level of conditioning, if at all; a CS-US interval of about 350 ms is optimal for conditioning (reviewed in Gormezano *et al.*, 1983). Similarly, in the case of pf-PC LTD, the extent of depression produced by a particular number of paired pf and cf stimulation varies with pf-cf stimulus interval (Ekerot & Kano, 1989).

The dependence of magnitude of pf-PC LTD on the interval between the parallel fibre and climbing fibre stimulation is usually explained in terms of "coincidence detection" - a cellular mechanism that is engaged only when a particular combination

of synaptic events occurs with particular temporal relationships. This mechanism could involve a rise in the postsynaptic Ca^{2+} concentration, in a manner similar to that of the Bienenstock-Cooper-Munro (BCM) rule (Bienenstock *et al.*, 1982). In the BCM rule, it is suggested that the direction and the amount of synaptic plasticity depends on the level of a postsynaptic biochemical signal, such as the Ca^{2+} concentration. This mechanism serves to change the synaptic efficacy in either direction. Whether the efficacy increases or decreases depends on the level of the signal with respect to a certain threshold. Such a mechanism involving postsynaptic activity explains the importance of the inter-stimulus interval. There would be a relatively narrow window of inter-stimulus interval that might lead to the required levels of postsynaptic activity leading to an increase in the synaptic efficacy, another window of inter-stimulus interval that would corresponds to a decrease, whereas the rest of inter-stimulus interval does not produce any significant plasticity, although the exact shape of the curve may differ from one type of synapse to another. In hippocampal pyramidal neurons, a Ca^{2+} rise above a certain threshold leads to a long-term potentiation of Schaeffer collateral inputs whereas Ca^{2+} levels below that threshold lead to a long-term depression. In the case of pf-PC synapse, however, the postsynaptic Ca^{2+} level rules appear to be a reversal of the BCM rule as it applies to neocortical or hippocampal neurons. High [Ca^{2+}] rise results in pf-PC LTD, whereas a moderate rise in Ca^{2+} leads to the induction of pf-PC LTP. This has been termed a reverse BCM rule (Coesmans *et al.*, 2004). *In vitro* investigation of the relationship between ISI and pf-PC LTD is complicated by the fact that it depends on other aspects of the stimulation protocol. For example, when single, rather than burst activation, of parallel fibre is used in conjunctive climbing and parallel fibre activations, the pf-PC LTD is induced optimally when the cf stimulation precedes pf

stimulation by 0-20 ms (Hartell, 1994; Karachot *et al.*, 1994; Sakurai, 1987). However, using burst pf stimulation during conjunctive stimulation, optimal pf-PC LTD induction occurs when the pf stimulation precedes the cf stimulation by about 100ms (Safo & Regehr, 2008; Wang *et al.*, 2000). In assessing the functional implications of these *in vitro* investigations, it should be noted that most are carried out in the presence of $GABA_A$ receptor blockers. The optimal temporal relationship is likely to be different when GABAergic inputs are active. For example, the PC Ca^{2+} rise during the climbing fibre response is reduced considerably by molecular layer interneuron activity (Callaway *et al.*, 1995). This may explain differences in the optimal induction protocols reported in pf-LTD induced *in vivo*, where activations of mossy, parallel and climbing fibres also activate the molecular layer interneurons (Ekerot & Kano, 1985).

In addition to the contribution of molecular layer interneurons, which are often ignored in *in vitro* analyses of pf-PC LTD induction, there are factors that make it difficult to compare directly the interstimulus intervals used in *in vitro* investigations of pf-PC LTD and in behavioural conditioning. One particular concern is to define the time-course for representation of peripherally presented stimuli in the cerebellum during *in vivo* work. Signals related to a tone CS go through many stages of processing from the point where the sound is transduced until they arrive at the relevant Purkinje cells. After the transduction of sound waves in the cochlea, the signal passed through numerous centres in the auditory pathway, including the cochlear nucleus, the superior olive, the inferior colliculus, the pontine grey and the granule cell layer of the cerebellum. At each stage of processing, the signals are likely to undergo a transformation. Thus, it is highly unlikely that CS-related signals in the

granule cell axons will have simple, square-pulse temporal profile of the presented CS. Perhaps unsurprisingly, exact relationships between optimal CS-US ISIs and learning rate do not transfer directly to pf-PC LTD induction parameters *in vitro*.

1.14.3. Time-course

The acquisition of conditioned NM responses is achieved in three distinct phases, which may be classified as (i) the training phase, where paired CS and US are repeatedly presented, (ii) the post-training, consolidation phase, where the memory is prone to disturbance, e.g. by a infusion of muscimol in the HVI up to 1 hour after an acquisition session (Attwell *et al.*, 2002) and (iii) the maintenance phase, where the memory that has been laid down is more stable than in the consolidation phase. Once learnt, the conditioned responses may be retained almost completely for at least 1 month (Schreurs, 1993) without reinforcement.

Does the induction of pf-PC LTD also have similar phases? The original description of pf-PC LTD reports that it was expressed slowly i.e. it appeared to develop slowly over about 30 minutes or longer after the conjunctive stimulation (Ito *et al.*, 1982) and the rate at which asymptotic depression is reached seems to vary with the protocol. Chemically, rather than synaptically, induced forms of pf- PC LTD that are induced using exogenous agonists seem to reach the maximum level of depression immediately after the induction protocol. Also during this early phase i.e. the phase immediately following induction protocol, an application of a protein synthesis inhibitor that blocks translation of proteins leads to a failure in pf-PC LTD induction (Karachot *et al.*, 2001). Therefore, there seems to be a phase in the development of pf-LTD that corresponds to establishing plasticity in a more permanent form, but which

may still be disrupted by certain manipulations. Once induced, the depression in the PC response to parallel fibre input has been routinely observed for 1 hour *in vivo* and in slice preparations (Ekerot & Kano, 1985; Sakurai, 1987). Reduced forms of pf-PC LTD have been recorded for longer periods. For example, a quisqualate-induced depression of Purkinje cell response to AMPA has been observed for 10 hours (Ito & Karachot, 1990). However, for the pf-PC LTD to underlie eyeblink conditioning, the depression may be expected to persist as long as the maintenance of conditioned responses. In trying to assess the longevity of pf-PC LTD, electrophysiological monitoring using frequent test stimuli to the pf, may not be ideal, because such test stimuli alone may induce the reversal of the depression. Stability of the experimental preparations, especially acute cerebellar slices, also limits the duration of investigation.

1.15. Extinction of conditioning

During extinction training, previously conditioned subjects are repeatedly presented with the CS alone. This results in a gradual decrease in the frequency of CRs and ultimately, CRs are no longer expressed in response to CS presentation. *In vitro*, weak stimulation of pf only leads to pf-LTP that can be of presynaptic or postsynaptic origin (Lev-Ram *et al.*, 2002; Salin *et al.*, 1996). At least in terms of the direction of the synaptic efficacy change, it is a reversal of an LTD and therefore has some properties similar to extinction. But behavioural extinction is not a simple reversal of learning, as indicated by spontaneous recovery and also by the phenomenon of savings. That is, following extinction, animals acquire conditioned responses more quickly than they originally had done from the naïve state. This improvement in learning is termed the savings. The amount of savings depends on the amount of

extinction training given and so there seem to be latent processes that are not fully revealed in the behaviour. Thus, any behavioural index of CR frequency may not, at any point in time, necessarily indicate the state of the neural mechanisms underlying learning because the process of extinction may be incomplete.

It has been suggested that the phenomenon of extinction can result from a two-stage learning mechanism, with one site having a slower time-course than the other. Mauk and colleagues have suggested this second site that has a slower time-course could be in the DCN(Mauk & Ohyama, 2004). However, a recent study (Jirenhed *et al.*, 2007) revealed that with paired mf stimulation (CS) and olivary stimulation (US), conditioned response-like pauses in PC activity were formed and these extinguished by presentation of mf stimulation alone, and a savings-like phenomenon was also observed. It seems unlikely, therefore, that this savings phenomenon would have involved the DCN. At the moment it is difficult to resolve where the second site may be, if it exists at all, but the behavioural observation of learning and extinction is likely to involve more than just reversals of synaptic plasticities at parallel fibre-Purkinje cell synapses. An investigation of alternating induction of pf-PC LTD and pf-PC LTP and analysis of the rates of initial and subsequent induction of these plasticities could help in answering whether acquisition and extinction depend upon changes at the same synapse or whether a second site is involved.

Even though the properties of the *in vitro* phenomenon of pf-PC LTD and behaviourally observed eyeblink conditioning do not seem to be identical, they share properties that make pf-PC LTD a possible cellular mechanism, which at least in part might underlie the association of CS and US during conditioning. How can we test

more directly whether pf-LTD is involved in cerebellum dependent learning? One approach is to disrupt the molecular pathways known to be involved in the induction of pf-PC LTD and observe the effect on cerebellum-dependent learning.

1.16. Molecular mechanisms of pf-PC LTD

1.16.1. Primary events

Since its discovery by Ito and colleagues (Ito & Kano, 1982; Ito *et al.*, 1982) there has been a wealth of investigations to elucidate the molecular mechanisms of pf-PC LTD induction. The majority of the early studies use the induction protocols originally developed by Ito – conjunctive stimulation of pf stimulation preceding cf stimulation by 0-20 ms, repeated every 1 second for 5 minutes. Based on these studies, it is suggested that there are three key events which act as external triggers for the induction of pf-LTD. Three key events are: (1) a glutamate release from the parallel fibres that activate AMPA receptors (those that eventually undergo modifications) and $mGlu_1$ receptors, (2) release of NO and the subsequent activation of the soluble guanylyl cyclase in the Purkinje cell cytoplasm, (3) activation of the climbing fibre, leading to the powerful Purkinje cell depolarisation and Ca^{2+} influx through voltage gated Ca^{2+} channels (see Ito, 2001 for review).

The sources and exact manner of glutamate and NO for triggering the above cellular events inside PCs are, however, not as clear as stated above. For example, the glutamate released from the climbing fibre may also activate $mGlu_1$ receptors (Dzubay and Otis, 2002). NO is thought to be produced in parallel fibre terminals. The production of NO is a Ca^{2+}-dependent process, as the enzyme involved in the

production of the molecule, the neuronal NO synthase, requires Ca^{2+}. The required Ca^{2+} rise may be associated with an activation of the presynaptic NMDA receptors (Casado *et al.*, 2002), though this is still debated. But there is also a suggestion that climbing fibres could also provide NO (Southam & Garthwaite, 1991).

1.16.2. Secondary events: crucial molecular events that follow the primary events

The pf-PC LTD is expressed when the AMPA receptors mediating parallel fibre EPSPs are appropriately modified. It is suggested that depression of pf-PC synapses is achieved by a decrease in the AMPA receptor density in the postsynaptic site (Matsuda *et al.*, 2000). This process results from the phosphorylation of the AMPA receptor, specifically the ser-880 residue of the GluR2 subunit, and involves PKC activation (Chung *et al.*, 2003). Whether or not PKC directly phosphorylates the receptor is not clear, as there are other kinases whose activation is involved in pf-PC LTD. For example the induction of pf-LTD by a PKC activator requires protein tyrosine kinase function (Boxall *et al.*, 1996). And another kinase, the alpha type calcium-calmodulin dependent kinase II (αCamKII) is also required for the pf-PC LTD induction (Hansel et al., 06). The phosphorylation of AMPA receptors reduces their affinity to the postsynaptic specialisation that contains postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) (PDZ domain). The AMPA receptors thus released from the grip of anchoring proteins are internalised in a clathryn-dependent manner (Wang & Linden, 2000). Phosphatases oppose the phosphorylation of AMPA receptors by kinases and, in the PC, phosphatases such as PP1, 2A/B act to increase the efficacy of pf-PC synapses (Belmeguenai & Hansel, 2005). Thus, the induction of pf-PC LTD

requires the inhibition of phosphatases; consistent with this, pf-PC LTD induction is blocked by an intracellular infusion of an activated PP2B.

How do the initial, external events described earlier culminate in the phosphorylation of the AMPA receptors? $mGlu_1$ receptor activation leads to the activation of one or more G proteins that include G_q and G_{11} (Hartmann *et al.*, 2004). The α-subunits of these G proteins activate phospholipase Cβ (Jiang *et al.*, 1994). When activated, this enzyme breaks down phosphatidylinositol-4,5-biphosphate (PIP2), which is a membrane constituent, into the two signalling molecules, IP_3 and diacylglycerol (DAG). PLCβ4 is the most widely expressed isoform, but PLCβ3 is also present in a subset of Purkinje cells that do not express PLCβ4 (Sarna *et al.*, 2006). IP_3, in conjunction with Ca^{2+}, activates IP_3 receptors on the endoplasmic reticulum and causes Ca^{2+} release from there. DAG, on the other hand, activates protein kinase C (PKC) leading to the phosphorylation of AMPA receptors as described above. DAG is further broken down into 2-arachidonylglycerol (2AG) by the action of DAG lipase (Bisogno *et al.*, 2003). 2-AG is an endocannabinoid that is thought to activate CB_1 receptors located presynaptically (Kreitzer & Regehr, 2002). The activation of CB_1 receptors is also required for the pf-PC LTD induction (Safo & Regehr, 2005), although at the moment it is not known how activation of presynaptic CB1 receptors leads to the expression of pf-PC LTD, which is postsynaptic.

The role of NO in the pf-PC LTD induction appears to be to target soluble guanylyl cyclase within the Purkinje cell; this enzyme catalyses the conversion of GTP into cyclic GMP (cGMP) - an activator of protein kinase G. One of the substrates of activated protein kinase G is G-substrate which, when phosphorylated by PKG,

inhibits protein phosphatases, especially PP1 and PP2A (Endo *et al.*, 1999). Thus by opposing the action of phosphatases, the NO signalling pathway augments LTD induction.

Ca^{2+} influences many of the steps described above. The release of Ca^{2+} from internal stores is one. The translocation of PKCα to the membrane, where, upon activation by DAG, it phosphorylates the AMPA receptors, requires a rise in $[Ca^{2+}]$ and the activity of many other enzymes, including PLCβ, is enhanced by Ca^{2+}. As mentioned earlier, the Ca^{2+} rise is strongly related to the coincidence detection i.e. a mechanism that reports a convergence of simultaneous parallel and climbing fibre activations. It is likely that this coincidence signal, in the form of high $[Ca^{2+}]$, is detected at multiple stages of signalling involved in the pf-PC LTD.

1.17. Mouse mutants, pf-PC LTD and their behaviour

If pf-PC LTD, as characterised *in vitro*, is a mechanism for memory storage in the cerebellum, then disruption of any of the molecular signalling pathways outlined above should impair cerebellar learning. To this end, several mutant mice have been developed, where molecules known to be important in pf-PC LTD have been specifically targeted, and the effects upon behaviour have been assessed. However, several factors must be considered in judging whether such studies reveal that some behaviour/learning processes observed truly depend upon pf-PC LTD: (i) To what extent are the investigated behaviours cerebellum-dependent? (ii) To what extent does the observed impairment stem from developmental anomalies in the system, rather

than as a direct result of the loss of pf-LTD? (iii) If little or no behavioural change is seen, have compensatory mechanisms developed as a result of the mutation?

1.18. Behavioural assessments of cerebellar functions in mice

Several types of behaviour have been used to assess cerebellar functions in mice. These include gait analysis (by recording footprints during walking), baseline performance and learning on a rotarod, delay eyeblink conditioning and vestibulo-ocular reflex (VOR) gain adaptation (de Zeeuw & Yeo, 2005). While all of the behaviours are likely to involve the cerebellum, it is not known to what extent they are dependent on the cerebellum. A deficit in motor performance could result from disruptions in any of the centres involved in these complex tasks, which is relevant if the experimental manipulations are not confined to the cerebellum, as is the case for global gene knockout (Table 1). Some of these tasks therefore may not allow appropriate assessments of cerebellar functions.

There are indications that mouse eyeblink conditioning is different from NM response conditioning in rabbits in several important ways. Since mice do not have a nictitating membrane, conditioning studies are of eyelid closure, controlled by the orbicularis oculi muscle that closes both lids and by the levator palpebrae that raises the upper eyelid. In contrast to the exclusively reflex behaviour of the NM response in rabbits, eyelid closure in mice and all other species is also under voluntary control. The acquisition and retention of conditioned eyeblink in mice is less robust than NM conditioning even in the normal subjects, which may relate to the additional voluntary control of the response. For example, mice show 20% responses before training, and

their maximum CR performance is approximately 80%; (Kishimoto *et al.*, 2002; Koekkoek *et al.*, 2003). In comparison, NM response conditioning in rabbits shows less than 6% responses before the training, and asymptotic CRs levels are close to 100% (reviewed in Gormezano *et al.*, 1983). Furthermore, unlike conditioned NM responses, conditioned eye blinks in mice are often poorly timed and they have a very short-latency component (Kishimoto *et al.*, 2002; Koekkoek *et al.*, 2003)

In the case of the mouse VOR and its gain modifications, wild-type animals show baseline VOR gain of less than 0.5 (i.e. eye movement cannot compensate for the head movement very well). The baseline VOR gain is even worse depending on the frequency of vestibular stimulation (de Zeeuw *et al.*, 1998; Kimpo *et al.*, 2005). Furthermore, gain of the VOR in the wild-type mice does not seem to adapt very well during training intended to induce gain changes. For example, a study by De Zeeuw *et al.* (1998) showed a gain increase after gain-up VOR training when the sinusoidal stimulus had a frequency of 0.4 Hz, but not other frequencies. The resulting VOR gain thus produced at this frequency was 0.38, which is an increase compared to the original gain of 0.21, but not enough to eliminate image motion on the retina. It is not clear to what extent these unusually low gain values may be specific to the head-fixed conditions that are used for most of these studies, nor whether they relate to the use of implanted eye coils for movement transduction.

1.19. Developmental abnormalities in cerebellar mutant mice

In most studies of cerebellar mutant mice to date, the gene disruption is present from the beginning of life so it is not surprising that some abnormalities in the development

of the nervous system are observed. Some problems are specifically associated with the global knockout of the genes, whereas others are common to both global and Purkinje cell specific knockout of the genes.

When the gene is disrupted globally, then, depending upon the normal pattern of expression of the gene in the brain, it becomes difficult or impossible to identify the cause of behavioural anomalies with an alteration in a specifically cerebellar function or with the function of a particular cerebellar neuronal type such as the Purkinje cell. For example, $mGlu_1$ receptors are found in numerous brain regions, including the thalamus, basal ganglia, cerebral cortex especially the hippocampus and olfactory bulb (Masu *et al.*, 1991). Numerous neuronal types in the cerebellar cortex, other than Purkinje cells, express the $mGlu_1$ receptor (see Figure 1.11). Therefore a global $mGlu_1$ receptor knockout has consequences for functions involving all of these structures.

Problems that are common to global and Purkinje cell-specific genetic interferences involve disruptions to the maturation of Purkinje cells and their synaptic inputs. The most common developmental anomaly of this kind is a persistent multiple climbing fibre innervation. This pattern of innervation is normal in the early stages of development but maturation of the olivocerebellar system is accompanied by the regression of weaker, competing climbing fibres so that, eventually, only one climbing fibre innervates each Purkinje cell. During normal development, most Purkinje cells complete this process approximately by the age of postnatal day 14 . However, in many mutant mice (see table 1), there is a persistent multiple climbing fibre innervation of Purkinje cells in the adult.

Multiple climbing fibre innervation is found in $mGlu_1$ receptor knockout mice, which are relevant to the work described in this thesis. Single climbing fibre innervation can be restored when $mGlu_1$ receptor is re-introduced in these global $mGlu_1$ receptor knockout mice, under the control of a Purkinje cell- and retinal bipolar cell-specific promoter, L7. Such $mGlu_1$ receptor -rescue mice are able to acquire conditioned external eyelid closure better than global $mGlu_1$ receptor knockout mice, indicating it is the $mGlu_1$ receptor function in Purkinje cells, not elsewhere, which is important for the behaviour. In a recent study, $mGlu_1$ receptors in Purkinje cells were conditionally knocked out in adult mice, that is, after normal, single climbing fibre innervation of Purkinje cell had developed. In these mice, the $mGlu_1$ receptor was initially expressed only in Purkinje cells but its expression could be turned off at any stage by an inducer, for example an analogue of tetracycline, doxycyline, that is included in the diet (Nakao *et al.*, 2007). When this Purkinje cell $mGlu_1$ receptor expression was turned off, the mice were reported to develop discoordination of movements. This study was useful in excluding the persistent multiple climbing fibre innervations as the only cause of motor deficits in mutant mice. However, because it took approximately 1 month to induce the conditional knockout, it is possible that the long-term removal of $mGlu_1$ receptor produced compensatory effects that dramatically modified Purkinje cell physiology. This also highlights a problem of establishing a strong causal link between pf-PC LTD and the behavioural deficit when many other cellular events are possibly affected. This can be appreciated by comparing different types of mice mutants that all lack pf-PC LTD but have different phenotypes. For example, pf-PC LTD is absent in both CB_1R and L7-specific, conditional $mGlu_1$ receptor knockout

mice. However, ataxia is reported only for the latter mice (Kishimoto and Kano, 2006; Nakao et al., 2007).

Target gene	Nature of disruption	Reported developmental abnormality	Effect on behaviour	References
$mGlu_1$ receptor	Global knockout	Multiple climbing fibre innervation remains; this can be "rescued" if functional mGlu1 expressed in Purkinje cells (L7-mGlu1 rescue)	Ataxic (gait analysis); impaired eyeblink conditioning. Normal eyeblink conditioning in L7mGlu1 rescue mice.	(Aiba *et al.*, 1994) (Conquet *et al.*, 1994)
$mGlu_1$ receptor	Conditional knockout in the background of L7- $mGlu_1$ receptor rescue	Multiple climbing fibre elimination allowed to occur before the induction of conditional knockout	Ataxic (gait analysis); impaired baseline performance on the rotarod, but slight improvement in the performance observed	(Nakao *et al.*, 2007)
PKC	PKCγ knockout	Multiple climbing fibre innervation remains; PKC isoforms upregulated Pf-PC LTD intact	normal	(Chen *et al.*, 1995)
PKC	Expression of PKC inhibitor under control of L7 promotor	Delayed elimination of multiple climbing fibre innervation	VOR gain adaptation impaired. Impaired eyeblink conditioning	(de Zeeuw *et al.*, 1998;Koekkoek *et al.*, 2003)
GluRδ2		Multiple climbing fibre innervation remains	Impaired eyeblink conditioning	(Kashiwabuchi *et al.*, 1995;Kishimoto *et al.*, 2001)
FMR-1		Homosynaptic pf-LTD		(Koekkoek *et al.*, 2005)
CamKIV	Global knockout	No report on the maturation of olivocerebellar system. The maintenance of pf-PC LTD is impaired.	Impairment in the retention of VOR gain adaptation in "gain-up" training. The amount of retinal slip is greater in gain-up, compared to gain-down training. The impairment in the retention of gain modification	(Boyden *et al.*, 2006)

			is frequency specific.	
αCamKII	Global knockout	Delayed elimination of multiple climbing fibre innervation	Impairment in VOR and OKR gain adaptaion	(Hansel *et al.*, 2006)
CB1	Global knockout	No report on cf-PC synapse maturation	Impaired delay but not trace conditioning. Effect on motor coordination is less severe compared to others, e.g. $mGlu_1$ receptor knockout mice	(Kishimoto & Kano, 2006)

Table 1.1

In summary, targeted disruption of genes in mice often results in serious developmental problems and the baselines of test behaviours in mice are often poorly characterised. To date, the gene knockout approach has not clearly demonstrated that pf-PC LTD underlies learning and memory processes in the cerebellum.

1.20. Experimental rationale: pharmacological inactivation of $mGlu_1$ receptor *in vivo*

In order to investigate the relationship between pf-PC LTD and cerebellum-dependent behavioural learning more directly, there is a need for a local, reversible disruption of pf-PC LTD induction during well-characterised, robust cerebellum-dependent learning. The experiments described in this thesis attempt to solve these difficulties by infusing an $mGlu_1$ antagonist in the lobule HVI of the rabbits during classical conditioning of NM responses.

The success of a pharmacological experiment relies on the antagonist acting effectively and that it has an expected action on the target but not other molecules i.e. the antagonist acts specifically. No compound perfectly fulfils these criteria but it is

possible to minimise unwanted effects of an antagonist *in vivo*, while retaining its intended effect on the target receptor. Selectivity refers to the preferential action of the antagonist on the target receptor over other, known targets or receptors. Potency is a measure of the concentration at which an antagonist is effective, such that the higher the potency of an antagonist, the lower the concentration of the antagonist required. An ideal antagonist should thus be as selective and potent as possible.

In order to discuss which $mGlu_1$ antagonist to be used in the project, some knowledge of the structure and function of the receptor and its relatives is beneficial. Potential targets of drugs, apart from the intended receptors, are often related in structure to the target receptors. In the case of $mGlu_1$ receptor, therefore, structurally related receptors are the other $mGlu_1$ receptors, as well as receptors that belong to the family 3 of G-protein coupled receptors (GPCRs; Figure 1.8).

Metabotropic receptors form a distinct class of glutamate receptors. Unlike ionotropic glutamate receptors, such as AMPA, kainate and NMDA receptors, metabotropic receptors are not ion channels.

Figure 1.7. G-protein coupled receptor families. (A) Generalised structure of GPCRs belonging to each family. N-terminal and carboxy-terminals are located in the extracellular and intracellular spaces, respectively, bridged by 7 transmembrane domains. Examples of ligands that activate receptors belonging to the group are listed next to the structures. Orange ovals represent ligands binding to the receptors. (B) A dendrogram showing the similarities in the amino acid sequence between different receptors. The distance at which branching occurs indicates the degree of similarity in the sequence i.e. earlier the divergence, less similar the amino acid sequence between the receptors. mGluRs belong to the family three and are related to Calcium (Ca^{2+}), $GABA_B$ and putative pheromone receptors coupled to Go (VR1, GoVN). A full list of mGluRs and their sequence similarity is given in Figure 9. From Bockaert & Pin (1999)

1.21. Metabotropic glutamate receptors – basic structure

Metabotropic glutamate receptors are transmembrane proteins. Each functional receptor is formed by 2 subunits of mGlu protein i.e. they form a dimer. Each subunit has three major domains: the extracellular, transmembrane and intracellular. Glutamate binds to the large extracellular domain, which corresponds to the N-terminal portion of the protein. Each unit is thought to fold in and out of the membrane, forming 7 transmembrane domains (TM) and 3 intracellular loops. Selectivity for G proteins, and thus the nature of the consequent signalling cascade is thought to be conferred by variations in the amino acid sequence of the 2nd intracellular loop (reviewed in De Blasi A. *et al.*, 2001). The intracellular domain – the C-terminal portion of the protein – is involved in the maintenance and modulation of the receptor. mGlu receptors are activated when glutamate binds to the extracellular domain, which causes the receptor to "hinge" (in a manner likened to that of the Venus Flytrap). This conformational change is thought to be transmitted to the intracellular domain via the transmembrane domain. The transmembrane domain contains sites to which exogenous compounds can bind to alter the functions of the receptors, in a manner that is non-competitive with glutamate binding.

1.22. Metabotropic glutamate receptors can be classified into groups

To date, 8 types of mGlu receptors have been cloned. $mGlu_1$ receptor was the first to be cloned (Masu *et al.*, 1991). All, except $mGlu_6$, are present in the cerebellar cortex. The 8 types of receptors can be classified into three groups – groups I to III. Group I mGlu receptors consist of $mGlu_1$ and $mGlu_5$, group II mGlu receptors consist of $mGlu_2$ and $_3$, and finally, group III mGlu receptors consist of $mGlu_4$, $_6$, $_7$ and $_8$. The groupings reflect similarities in their amino acid sequences, signalling pathways and

pharmacology (Figure 1.8B). For example, $mGlu_1$ and $mGlu_5$ have about 60% sequence similarity. Often such similarity in the amino acid sequence results in some antibodies failing to discriminate between receptor types (e.g. an antibody against the C-terminal portion of the $mGlu_2$ receptor also recognises that of $mGlu_3$ receptor that has 80% sequence similarity; Ohishi *et al.*, 1994). Similarities and differences in the signalling pathways are seen in the types of G protein to which the receptors couple, e.g. Group I mGlu receptors couple to Gq/11 proteins whereas Group II and III mGlu receptors couple to Gi/o. Hence, activation of group I mGlu receptors results in an increase in intracellular [IP3], whereas activation of group II and III mGlu receptors results in a decrease in [cAMP] via inhibition of adenylyl cyclase. Similarities in mGlu receptor pharmacology can be seen in the common actions of some agonists e.g. $mGlu_1$ and $_5$ can be activated by DHPG, mGlu $_2$ & $_3$ and mGlu $_{4, 6, 7}$ and 8 by DCG-IV and L-AP4, respectively (see Figure 1.8B). Such similarities between mGlu receptors that belong to the same group mean that several types are likely to be affected during a pharmacological manipulation. Indeed, for $mGlu_1$ antagonists, an action on $mGlu_5$ is almost always present. While it may be difficult to synthesise an $mGlu_1$ antagonist that completely lacks action on the $mGlu_5$ receptor, a wide difference in the potencies for $mGlu_1$ and $mGlu_5$ receptors can give enough selectivity to the antagonist.

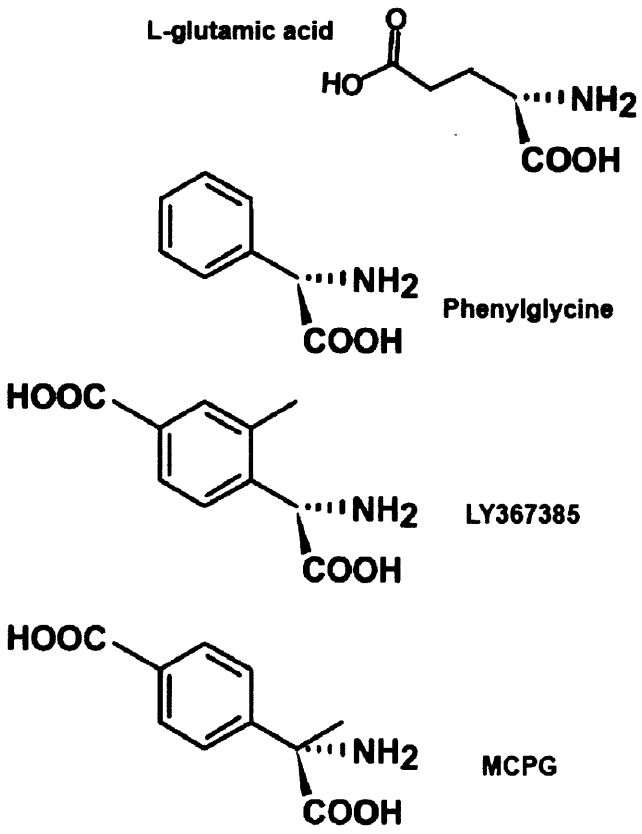
Figure 1. 8. mGlu receptor groups. A dendrogram on the left shows the degree of sequence similarity between 8 mGlu receptor types and parathyroid calcium sensor (PCaR1). mGlu receptors belonging to the same group have greater sequence similarities than with those from other groups. The mGlu receptors within each group also have similar transduction mechanisms and are activated by group-selective agonists. DmGluRA Drosophila mGluRA. (A) adapted from Conn & Pinn, 1997; (B) from IUPHAR receptor database.

1.23. Non-competitive antagonism of $mGlu_1$ receptor activation

Pharmacological block of mGlu receptors can be achieved in two ways. One method is by direct i.e. competitive interference with the glutamate binding using compounds that bind to the glutamate-binding site in a competitive manner. Such competitive

antagonists are mostly analogues of the amino acid phenylglycine, which is structurally similar to glutamate. Examples of competitive $mGlu_I$ antagonist are LY367385 (also known as (+)-4C2MPG) and MCPG (Figure 1.10). MCPG is not $mGlu_I$ specific but acts on group I & II mGlu receptors. Their dose-response curves can shift depending on the concentration of agonist (e.g. [glutamate]), so that a higher concentration of the antagonist is required to block $mGlu_I$ receptor when the agonist concentration is higher.

Figure 1.9. Structure of L-glutamate (glutamic acid) and phenylglycine and its derivatives LY367385 and MCPG.



The second way to interfere with the receptor function is via a site other than the glutamate binding site. For $mGlu_1$ receptor, the transmembrane domain (TM), especially the 7th TM, contains such a site. Pharmacological agents that disrupt mGlu receptor signalling by acting at this site do so independently of glutamate binding and, are, therefore, classed as non-competitive antagonists.

Which type of antagonist is more desirable for behavioural experiments? There is an advantage in using a non-competitive antagonist, because we can base our *in vivo* antagonist dosage on *in vitro* assessments. That is, the effective antagonist concentrations for use *in vivo* are based on our assessment against an artificially evoked $mGlu_1$ response in slices – that evoked by 10 pulses stimulation of pf at 100 Hz. However, the conditions, especially the concentration of glutamate reached at the relevant $mGlu_1$ receptors, may differ between the two experimental conditions: glutamate concentration at the receptors is dependent on the amount of incoming neural activity. In the particular case of interest here, this activity relates to the firing pattern of parallel fibres. Studies on granule cell activities are just emerging and so far, they suggest that granule cells *in vivo* can generate action potentials at frequencies as high as ~900 Hz (Jorntell & Ekerot, 2006). This uncertainty makes the bath assessment of competitive antagonist for use *in vivo* less reliable than for non-competitive antagonist, whose action is independent of glutamate concentration.

1.24. Possible consequences of $mGlu_1$ receptor block other than the block of pf-PC LTD induction

Given the involvement of $mGlu_1$ receptor in various cellular mechanisms, an infusion of mGlu1 antagonist in the cerebellar cortex is likely to produce a multitude of effects,

in addition to blocking pf-LTD induction. Antagonism of $mGlu_1$ receptors *in vivo* is not likely to produce a direct action on the fast transmission mediated by ionotropic glutamate and GABA receptors because an $mGlu_1$ receptor antagonist does not act on the AMPA, NMDA and $GABA_A$ receptors. Therefore, during classical conditioning, PCs are still likely to modulate firing rate in some way to the CS and US presentations in the presence of an $mGlu_1$ antagonist. However, it is not known whether an $mGlu_1$ antagonist could change the exact manner of PC spike modulation to its inputs as well as the basal firing rate. One manner in which an mGlu1 antagonist may interfere with synaptic transmission is by blocking the production of endocannabinoids (eCB) which usually accompanies $mGlu_1$ receptor activation. An activation of the presynaptic CB_1 receptors by eCB leads to inhibitions of glutamate and GABA release, which lasts for a few 10's of milliseconds. Since $mGlu_1$ receptor are activated optimally following burst activity in the parallel fibre input (Batchelor et al., 94), an $mGlu_1$ antagonist may therefore affect fast synaptic transmission following burst activity in the parallel fibres.

In modelling studies of classical conditioning, it was suggested that the $mGlu_1$ signalling cascade that results in IP_3 and DAG concentrations increases underlies the generation of conditioned responses, not by its contribution to pf-PC LTD induction but rather by directly producing well-timed pauses in the simple spikes of Purkinje cells. This idea was inspired by the similarity in the time-course of local Ca^{2+} rise after $mGlu_1$ receptor activation to the optimal CS-US interval for classical conditioning (Fiala *et al.*, 1996; Steuber & Willshaw, 2004). In these hypotheses, Ca^{2+} release from intracellular stores mediated by IP_3R was suggested to activate Ca^{2+}-activated K channels (K_{Ca}), thereby hyperpolarising the cell. To explain how well-

timed responses could be generated adaptively using this mechanism, it was suggested that a population of Purkinje cells can possess a spectrum of delays between the time glutamate is released and the Ca^{2+} concentration rises (Fiala *et al.*, 1996). Those Purkinje cells that have an appropriate delay would activate PKC at the same time as the activation of PKG, which, in their model, corresponds to the arrival of US-related signal. The coincident activation of PKC and PKG was suggested to result in a phosphorylation of the K_{Ca} and only when the K_{Ca} is phosphorylated can the local Ca^{2+} rise be expressed as a hyperpolarisation. Steuber *et al.* (2007) suggested a similar role for the $mGlu_1$ receptor, but with each Purkinje cell adaptively controlling the timing of PKC activation following glutamate release by altering the concentration of $mGlu_1$ receptor. Such change in the $mGlu_1$ receptor concentration at the synapse was suggested to occur when PKC and PKG are active at the same time. While these hypotheses are attractive, there is currently no experimental evidence suggesting that a pause in PC firing follows $mGlu_1$ receptor activation and that this is the mechanism generating learned output. These hypotheses can, however, easily be tested as will be described in chapter 4.

There is also a "parallel" signalling pathway that is activated by the $mGlu_1$ receptor, which does not require activation of G proteins and controls slow depolarising potentials in Purkinje cells ($mGlu_1$ EPSP). The $mGlu_1$ EPSP is observed in slice in pharmacological isolation i.e. in the absence of AMPA, $GABA_A$ and $GABA_B$ receptor blockers. The inward current underlying this response is thought to be mediated by a non-specific cation channel, namely TRPC1 (Kim *et al.*, 2003). This response is potentiated for tens of seconds after climbing fibre activity (Batchelor & Garthwaite, 1997). This mechanism may be involved in integrating parallel fibre and climbing

fibre signals for phenomena that have integration time-courses different from the optimal pf- and cf- intervals for pf-PC LTD induction. However, it is not known whether the $mGlu_1$ EPSP modifies the pattern of PC simple spike firing *in vivo*, or whether the depolarisation produced contributes to long term modifications of PC properties.

In considering the application of $mGlu_1$ antagonists to the cerebellar cortex, thought must be given to possible actions other than that on parallel fibre synapses. On Purkinje cells, $mGlu_1$ receptors are known to be located also at climbing fibre – Purkinje cell synapses, as revealed by immunohistochemical analyses at EM level suggest (Nusser *et al.*, 1994). The $mGlu_1$ receptors are found perisynaptically and so burst activity of the climbing fibre may lead to preferential activation of this receptor, as suggested by studies on pf synapses (Batchelor *et al.*, 1994). The $mGlu_1$-EPSP recorded following single pulse climbing fibre stimulation is present but weak though it is significantly enhanced at higher (20 Hz) stimulus frequency and/or in the presence of a glutamate uptake blocker (Dzubay & Otis, 2002). Thus, the climbing fibre-Purkinje cell synapse is a potential target of $mGlu_1$ antagonists. Whether or not an $mGlu_1$ antagonist alters the waveform of climbing fibre responses will be a focus of investigation in Chapter 2.

As it can be seen in Figure 1.11, the $mGlu_1$ receptor is present in other cerebellar cortical neurons, especially in the stellate and basket cells. These molecular layer interneurons respond with slow depolarising potentials when the $mGlu_1$ agonist DHPG is applied or in response to burst parallel fibre stimulation in pharmacological isolation (Karakossian & Otis, 2004). In these neurons, $mGlu_1$ receptor activation, in

conjunction with NMDA receptor activation, leads to an endocannabinoid release that inhibits glutamate release from the parallel fibre terminals (Beierlein & Regehr, 2006). Furthermore, $mGlu_1$ receptor activation is required for a presynaptic form of LTD at pf-stellate (aspiny) cell synapse (Soler-Llavina & Sabatini, 2006). Therefore the $mGlu_1$ receptor seems to contribute in many ways to the physiology of molecular layer interneurons.

A

Neuronal type	Expression pattern (and the splice variant) as revealed immunohisochemically	Cellular functions
Purkinje cell	Surrounding the post synaptic density on dendritic spines (Baude *et al.*, 1993)	Required for the maturation of climbing fibre synapses. Its activation leads to eCB synthesis and short term depression of IPSCs and EPSCs; $mGlu_1$ receptor activation required for the pf-LTD induction
Stellate/basket cells	Observation at electron microscopic level not available	$mGlu_1$ receptor activation required for induction of pf-stellate cell LTD (presynaptic form; Soler-Llaviana & Sabatini, 2006) An agonist application results in a slow EPSP; pharmacologically isolated $mGlu_1$ EPSP also detected following burst pf stimulation (Karakossian &Otis, 2004) Joint activation of NMDA&mGlu1Rs leads to eCB production (Beierline and Regehr 2006)

Golgi cell	Only a subset of Golgi cells express mGlu1R (Baude *et al.*, 1993)	Not clear; Golgi cells do not seem to release endocannabinoid after $mGlu_1$ activation (Beierline and Regehr, 2006)
Granule cell	Expressed in a subset of granule cells ($mGlu_1a$; Baude et al., 1993)	
Unipolar brush cell	Dense expression in the dendrites (Takacs *et al.*, 1999)	

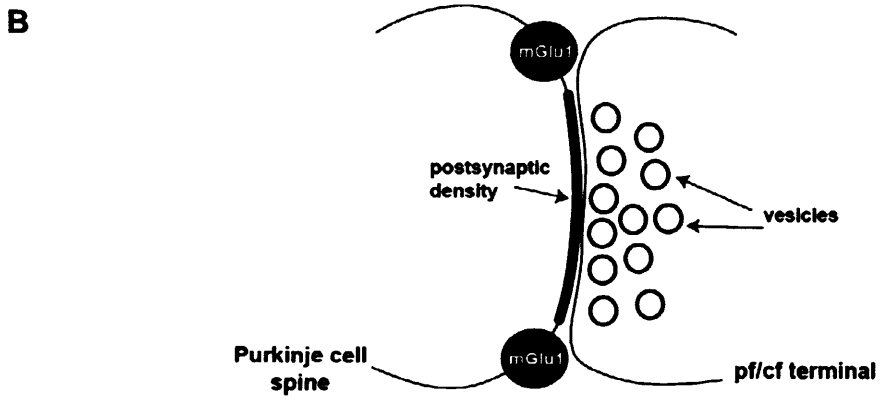


Figure 1.10. Cellular and subcellular distribution of $mGlu_1$ receptors in the cerebellar cortex. (A) Cerebellar cortical neurons that express **$mGlu_1$ receptor** and the possible cellular functions bestowed by the receptor. (B) Subcellular distribution of **$mGlu_1$ receptors** at the excitatory synapses on Purkinje cell spines. mGlu1R receptors are found immediately surrounding the postsynaptic density.

1.25. Conclusion

As with any technique, the pharmacological approach can have limitations. No single experiment can definitely link pf-PC LTD with cerebellum-dependent behavioural learning. However, a carefully designed experiment that investigates the relationship between one essential component mechanism of pf-PC LTD and behavioural learning is useful. If it can show that the mechanism is common to both, then it would join a body of evidence consistent with the idea that pf-PC LTD is a reasonable model of the

plasticity underlying the behaviour. A dissociation of underlying mechanisms should lead us to reconsider that hypothesis.

Chapter 2

2.1. Introduction

For the reasons pointed out in chapter 1, the ideal $mGlu_1$ antagonist for use *in vivo* would have a specific, selective and non-competitive action at $mGlu_1$ receptors. CPCCOEt was the first subtype selective, non-competitive $mGlu_1$ antagonist to be synthesised (Annoura *et al.*, 1996; Figure 2.1) and, at the outset of this study, was the only one available. In the work reported in this chapter, the aim was to use *in vitro* experiments to characterise the actions of CPCCOEt in the cerebellar cortex and thus determine its suitability of CPCCOEt for the subsequent *in vivo* work.

Figure 2.1. Structure of the non-competitive mGlu1 antagonist CPCCOEt.
From (Annoura *et al.*, 1996).

The selectivity of CPCCOEt for $mGlu_1$ over other mGlu receptor subtypes has been tested against human, heterologously-expressed $mGlu_{2, 4a, 5, 7b, 8a}$ in mammalian cell lines (Litschig *et al.*, 1999). CPCCOEt caused a minor potentiation (~35%) of forskolin-induced increase in cAMP concentration in cells expressing $mGlu_{7a}$, although CPCCOEt does not activate $mGlu_7$ on its own or inhibit the action of an $mGlu_7$ agonist (Litschig *et al.*, 1999). CPCCOEt did not affect signalling linked to other mGlu receptors at the concentration tested (100 μM). Importantly, it is selective for $mGlu_1$ over $mGlu_5$. In Chinese hamster ovary cells expressing either $mGlu_{1b}$ or

$mGlu_{5a}$, CPCCOEt inhibited the action of the agonist quisqualate with IC_{50}s of 9.9 μM and 933 μM, respectively (Casabona *et al.*, 1997).

What is the evidence that CPCCOEt acts in a non-competitive manner at the $mGlu_1$ receptor? CPCCOEt does not produce a rightward shift in the agonist-response relationship, but rather produces a concentration-dependent decrease in the maximum response (Hermans *et al.*, 1998). Analyses showed that EC_{50} and the Hill coefficient of the $mGlu_1$ agonists did not change in the presence of CPCCOEt. These are characteristics of a non-competitive antagonist. In addition, [^{3}H]-glutamate binding to $mGlu_1$ receptors is not displaced by CPCCOEt (Litschig *et al.*, 1999). Site-directed mutagenesis indicates that CPCCOEt binds to the 7th transmembrane domain (TM7), quite some distance away from the glutamate binding site in the large extracellular domain containing the N-terminal (Litschig *et al.*, 1999).

Is CPCCOEt effective on native $mGlu_1$ receptors? It has been used reliably as an $mGlu_1$ antagonist in many brain areas over the last decade. In the case of cerebellar Purkinje cells, CPCCOEt antagonises their responses to an exogenous agonist, either as photolytically released 'caged' glutamate (Canepari *et al.*, 2001) or as local application of a selective $mGlu_1$ agonist (Yamakawa & Hirano, 1999). Furthermore, the synaptic activation of $mGlu_1$ receptors following burst stimulation of parallel fibres is also effectively inhibited by CPCCOEt (Batchelor *et al.*, 1997; Miniaci *et al.*, 2001).

The planned behavioural studies will use rabbits but the effects of CPCCOEt on $mGlu_1$ have never been reported in this species. This might not be a trivial distinction.

Unlike the glutamate binding site, no endogenous agonist has been discovered for the CPCCOEt binding site. If this binding site does not have a physiological role in receptor functions, there may not be the same degree of evolutionary pressure for its conservation. The importance of conservation of binding sites for drug action can be seen in the case of another non-competitive antagonist, 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile (EM-TBPC). This antagonist is effective on rat $mGlu_1$ receptors, but not on human $mGlu_1$ receptors, even though their overall amino acid sequence homology is 91% and its action on human $mGlu_1$ receptors can be restored by replacing just one amino acid (Malherbe *et al.*, 2003). So, before using CPCCOEt in behaving rabbits we wished to test whether rabbit $mGlu_1$ is sensitive to this compound. In the absence of heterologously expressed rabbit $mGlu_1$ receptors (not yet cloned) we chose to characterise CPCCOEt's action on $mGlu_1$ responses in rabbit cerebellar slices.

As described in Chapter 1, the climbing fibre synapse is also a target for CPCCOEt action. A previous electrophysiological study using a competitive mGlu receptor antagonist, MCPG, reported that $mGlu_1$ receptor activation was not detected following a single pulse activation (Batchelor & Garthwaite, 1997). Given the potentially high glutamate concentration following climbing fibre activation and resulting possibility of a displacement of competitive $mGlu_1$ antagonist, we wished to confirm this using a non-competitive antagonist. This was to resolve the possible cellular contribution of climbing fibre activation in the pf-PC LTD. The main role of climbing fibre activation in pf-PC LTD is thought to be to raise the intracellular Ca^{2+} concentration and the $mGlu_1$ receptor activation is classically thought to occur following pf activations. If, however, $mGlu_1$ receptor is activated following climbing

fibre activation then not only should this be recognised but also it should be noted that a pharmacological block of $mGlu_1$ receptor may lead to a reduction in the Ca^{2+} influx during climbing fibre response by blocking $mGlu_1$ EPSPs.

Therefore, the aim of the experiments in this chapter was to test the effectiveness of CPCCOEt on synaptically activated $mGlu_1$ responses in rabbit using cerebellar slices, and to test if CPCCOEt modifies climbing fibre responses in rabbit Purkinje cells.

2.2. Methods

2.2.1. Preparation of rabbit cerebellar slices

Male New Zealand White rabbits (~1.5 kg) were killed with an overdose of pentobarbitone (Euthatal, 1.5 ml i.v.). To obtain high quality slices in rabbits, we found that it was necessary to transcardially perfuse with cold (~5 °C) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18, D-glucose 11, pH 7.4 when equilibrated with mixture of CO_2 and O_2 (5:95%). In order to perform transcardiac perfusion the thorax was cut open to expose the internal organs. The descending aorta was clamped with a haemostat to concentrate the perfusion to the dorsal aorta, and the right ventricle was opened to insert an outlet of a perfusion pump. The outlet of the perfusion system was clamped in the dorsal aorta. The left atrium was opened before starting the perfusion, which lasted for about 30 seconds. After the perfusion, the skull was rapidly opened to reveal the cerebellar vermis, which was carefully excised and removed. One of the parasagittal cut surfaces was glued to the platform of a vibatome (TPI 1000+, Intracel, Royston, UK) to make 250 μm-thick parasagittal slices. The slices were transferred to

a recovery chamber containing circulating aCSF, equilibrated with CO_2/O_2 mixture (5:95%) at room temperature (~25°C).

2.2.2. Preparation of rat cerebellar slices

Slices from rats were produced in essentially the same way as for rabbits (above) with the exception that transcardiac perfusion with cold aCSF was not performed. Male Sprague-Dawley rats (28 days old) were stunned and decapitated according to UK legislation (Animals (Scientific Procedures) Act 1986). The head was rapidly cooled by pouring cold aCSF onto it. The caudal end of the skull was cut open to expose the cerebellum which was again doused with cold aCSF. The peduncles were detached with scissors and the cerebellum was taken out and placed on a cold block where parasagittal incisions were made with a razor blade. One of the cut surfaces was glued to the platform of the vibrotome and 250 μm-thick parasagittal slices were made as described above.

2.2.3. Recording

Cerebellar slices were placed in a recording chamber, held down by a slice weight and perfused continuously with warm (~30 °C) aCSF. Purkinje cell somata were visually identified using differential interference microscopy (Leica, x40 water immersion objective). Purkinje cell somata were easily identified by their size and their position in a monolayer situated between the granule cell and molecular layers. 'Healthy looking' (shiny and no obvious dendritic swelling) Purkinje cells on or near the surface of slices were chosen for recording. The identity of the neuron was confirmed electrophysiologically. Purkinje cells are spontaneously active at resting membrane potential and, when held at -70 mV, respond to hyperpolarising current injection with

a "sag" in the membrane potential due to activation of I_h (see Figure 2.2b). In some cases, additional evidence confirming the identity of the cells as Purkinje cells was provided by evoking their characteristic climbing fibre responses (Figure 2.2d). Recording electrodes were made by pulling borosilicate glass (WPI; o.d. 1.5 mm, i.d. 0.84 mm, 7740 borosilicate glass with filament) with a resistance of about 3 MΩ when filled with a solution that contained (in mM): KCH_3SO_3 135; NaCl 4; KCl 10; EGTA 1; HEPES 10; NaGTP 0.4 and MgATP 4; 290 mOsmol kg^{-1}, pH 7.3 adjusted with KOH. The same internal solution was used for current-clamp (Axoclamp 2A, Molecular Devices, Sunnyvale, CA, USA) and voltage-clamp recordings (Axopatch 200B, Molecular Devices). While it is impossible to avoid disrupting the intracellular environment during whole cell patch clamp recordings, compositions of an internal solution should be to minimise interference with the intracellular environment. Following is a retrospective review of composition of the internal solution used in chapters 2 and 3. The largest component of the intracellular solution used here was potassium methanesulphonate. Organic anions are reported to improve the stability of currents than simple inorganic anions such as chloride ions. However, organic anions commonly used in intracellular solutions are not without problems, for example, methylsulphate and gluconate ions are both reported to interfere with potassium currents (Zhang *et al.*,1994; Velumian *et al.*, 1997; Kaczorowski et al., 2007). Methanesulphonate is less well explored. EGTA was chosen as the Ca^{2+} buffer because of its preference for Ca^{2+} over Mg^{2+} at pH 7.3 (Cheng &Cheng, 1974). A high concentration of EGTA was used as Purkinje cells are neuronal types (Fierro & Llano, 1996) probably due to high expression levels of calbindin and paravalbumin (reviewed in Hartmann & Konnerth, (2005)). However, because Ca^{2+} binding properties of EGTA are not exactly the same as those of endogenous Ca^{2+} buffers, it is

expected to disrupt the dynamics of intracellular Ca^{2+} signals. The amount of EGTA used in this internal solution may be higher than optimal (Kosaka *et al.*, 1993). This, together with 4 mM Mg^{2+}, which inhibits Ca^{2+} -dependent phenomena (Pessah *et al.*, 1987) due presumably to its ability to compete for the binding site, may cause additional interference with intracellular Ca^{2+} signalling.

2.2.4. Synaptic Stimulation

Stimuli were applied as rectangular voltage steps (100 μs wide and 5~30 V amplitude; DS2, Digitimer, Welwyn Garden City, UK) through a glass pipette which had resistance of approximately ~1 MΩ when filled with aCSF. To stimulate parallel fibres, the tip was placed in the molecular layer, about 100 μm below the surface of the slice. To stimulate a climbing fibre, the electrode was placed in the granule cell layer, in the vicinity of the recorded Purkinje cell soma (see Figure 2.2a). Clean climbing fibre stimulation was ensured by just-threshold stimulation, such that there was no inadvertent response in the Purkinje cell when a climbing fibre failed to activate (Figure 2.2c). Climbing fibre responses and fast parallel fibre EPSPs were recorded in the presence of bicuculline (30 μM). To obtain a pharmacologically isolated EPSP mediated by $mGlu_1$ ($mGlu_1$-EPSP), parallel fibres were stimulated 8-10 times at 100 Hz, every 60 seconds, in the presence of (in μM); NBQX (10), bicuculline (30), D-AP5 (30), CGP55485 (10) in the aCSF to block AMPA, $GABA_A$, NMDA and $GABA_B$ receptors, respectively.

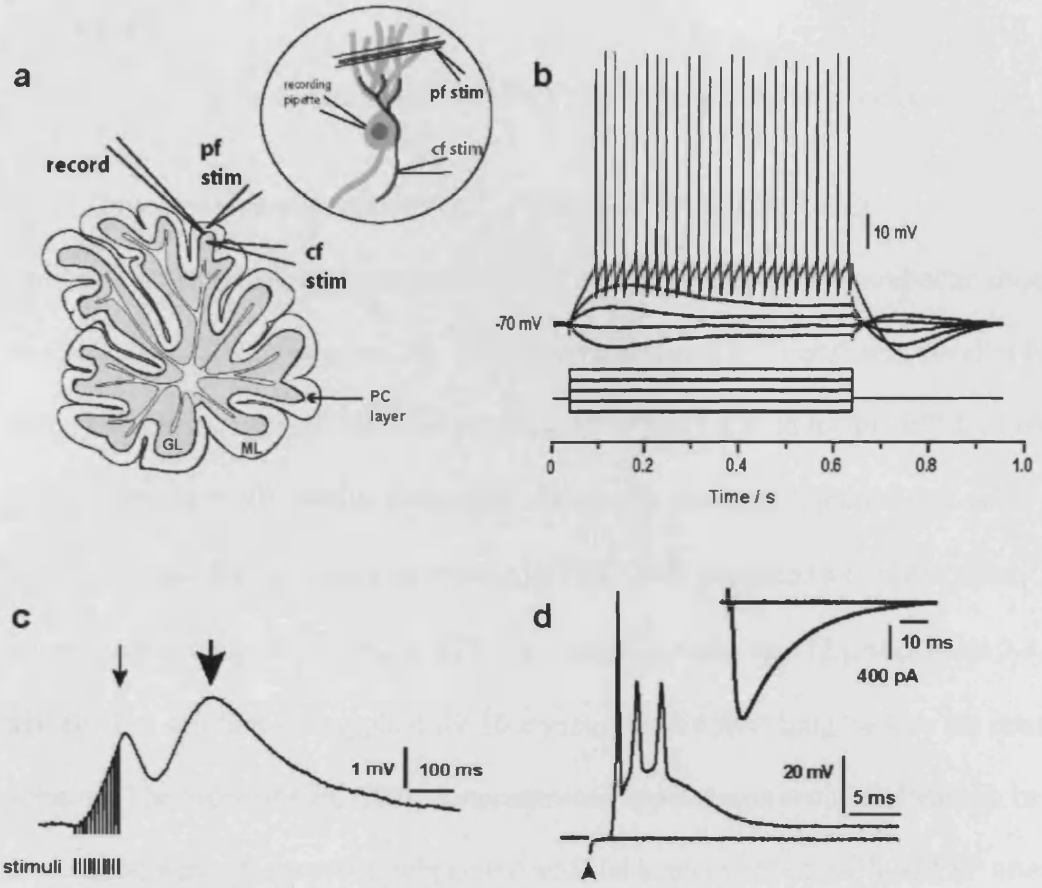


Figure 2.2. Experimental setup and sample electrical responses. (a) Parasagittal slice of mid-vermal cerebellum, depicting the locations of recording and stimulating electrodes relative to the Purkinje cell monolayer. GL = granule cell layer, ML = molecular layer. (b) An example of current-clamp recording from a Purkinje cell, in response to steps of current injection, from a holding potential of -70 mV. Each current step increases by 0.05 nA. (c) An example of a pharmacologically isolated mGlu1-EPSP. Vertical lines represent stimuli applied to parallel fibres. There is a short-lasting depolarisation that builds up during stimulation (thin arrow), which precedes the mGlu1-EPSP (its peak marked by a thick arrow). (d) Examples of all-or-none climbing fibre responses. The main trace shows a response recorded in current-clamp. The arrow head represents the time of stimulation. The inset shows the climbing fibre EPSC in voltage-clamp, recorded in the presence of 300 nM NBQX to improve the quality of clamp.

2.3. Results

Pharmacological characterisation of CPCCOEt in rabbit Purkinje cells

2.3.1. Concentration-dependent block of $mGlu_1$-EPSP by CPCCOEt

Current-clamp recordings were made from Purkinje cells in rabbit cerebellar slices. Pharmacologically isolated $mGlu_1$-EPSPs were obtained by stimulating parallel fibres with short bursts, applied every 60 seconds. The slow, delayed $mGlu_1$-EPSP peaked at 531 ± 15 ms (n = 10) relative to the first stimulus in the burst (Figure 2.2c). After 10 minutes of baseline recording, perfusion of CPCCOEt produced a concentration-dependent decrease of the $mGlu_1$-EPSP with an IC_{50} value of ~12 μM (Figure 2.3A and B). The solution was applied for 10 minutes before switching back to the control solution. The order of CPCCOEt concentrations applied was semi-randomised. In some cells, where there was a substantial and stable recovery of $mGlu_1$-EPSP after washing with control solution, a second application of CPCCOEt was performed. Therefore some cells generated more than one data point.

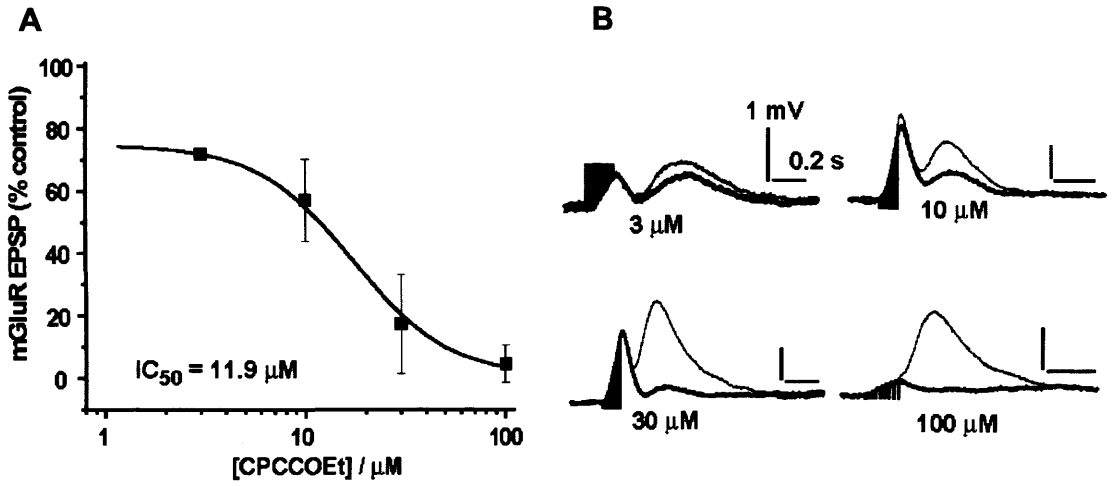


Figure 2.3. Concentration-dependent antagonism of mGlu EPSP by CPCCOEt. (a) amplitude of mGlu EPSP remaining after applications of CPCCOEt is expressed as % of that during the control period, and plotted against the concentration of CPCCOEt applied (n = 3 for each point). The IC_{50} value was interpolated from the sigmoidal fit to the data. (b) Examples of mGluEPSP before (thin line) and after (thick line) the application of CPCCOEt. Concentrations of CPCCOEt applied are indicated below each example

2.3.2. CPCCOEt enhancement of climbing fibre response

Current-clamp recordings of climbing fibre responses were obtained from Purkinje cells in rabbit cerebellar slices. A typical climbing fibre response was observed rising about ~1 ms after the stimulus artefact, which corresponds to the synaptic delay. The response was composed of a large initial spike, followed by 1-4 smaller spikes which were separated by ~2 ms. This 'spiking phase' lasted for 10-15 ms (Figure 2.4) and was followed by a prolonged depolarisation lasting up to 100 ms which was often followed by an after-hyperpolarisation. The waveform varied from cell to cell but for a particular cell, the trial-to-trial variability of the climbing fibre response was

relatively small, as can be seen in the distribution of spike/spikelet timing (Figure 2.4A).

Unexpectedly, application of CPCCOEt at a concentration which caused a near maximal block of the $mGlu_1$-EPSP (100 μM) caused the spiking phase of the climbing fibre response to be enhanced. This sometimes led to an additional spikelet (Figure 2.4), which fully reversed on washout. In other cells application of CPCCOEt enhanced the depolarising phase of the climbing fibre response without generating an additional spike.

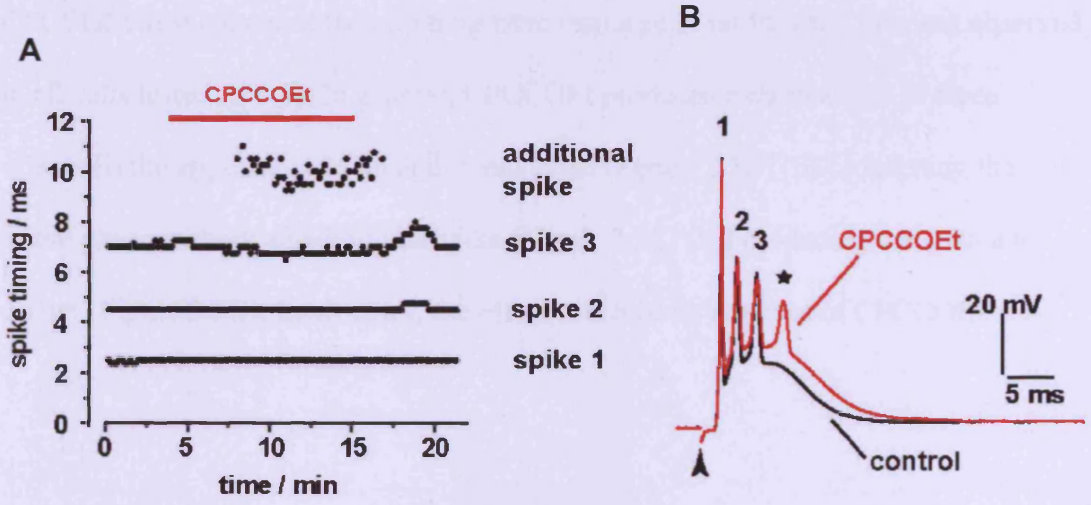


Figure 2.4. CPCCOEt enhancement of complex spike response in rabbit Purkinje cell. (A) Each dot represents the peak of each spike in a climbing fibre response (spike-timing plot). This example climbing fibre response had 3 spikes during each climbing fibre response during the control period. An application of CPCCOEt (100 μM; red bar) resulted in an additional spike. This effect reversed on wash out. (B) Example of climbing fibre responses from the same cell in (A). CPCCOEt produced an enhancement of the spiking phase of the climbing fibre response (red) compared to the control conditions (black). The additional spike is marked with a star. Arrowhead represents the time at which stimuli were applied. Spike-timing was measured from the stimulus artefact.

The mechanism underlying the enhancement was unclear and further investigation was necessary. For reasons of cost and ease of preparing slices, and to examine the possible generality of the finding, these further studies used rat cerebellar slices.

Investigation of the CPCCOEt effect in rat Purkinje cells

2.3.3. CPCCOEt effect on climbing fibre response was replicated in rat Purkinje cells

Current-clamp recordings of climbing fibre responses were made from Purkinje cells in rat cerebellar slices, using protocols similar to those described in section 2.1. CPCCOEt also enhanced the climbing fibre response in rat PCs and this was observed in all cells tested (n = 6). In general, CPCCOEt produced combinations of three effects (i) the appearance of an additional spike (Figure 2.5B) (ii) broadening the initial phase without an additional spike (Figure 2.5C) (iii) producing the spikelets earlier (Figure 2.5D). In all cases, the effect reversed on removal of CPCCOEt.

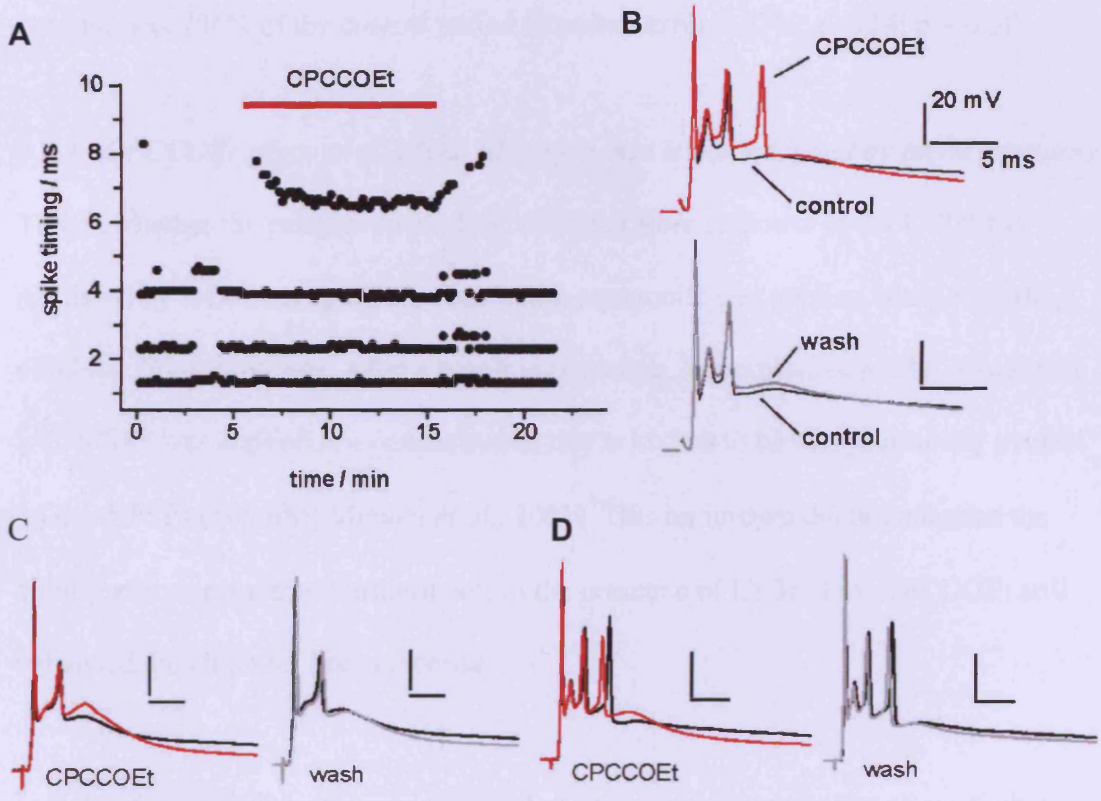


Figure 2.5. CPCCOEt effect on climbing fibre responses. (A) spike-timing plot of climbing fibre responses recorded in Purkinje cells from rat cerebellar slices. (B)-(D) example traces showing different forms of enhancement produced by CPCCOEt, resulting in an additional spikelet (B), broadening of the initial depolarising phase without an additional spikelet (C) and decrease in the inter-spikelet interval (D).

On application of CPCCOEt, no systematic change in the passive membrane properties was observed. The apparent input resistance, measured by the steady-state change in the membrane potential in response to an injection of hyperpolarising step current, was 104% of the control period (standard error = 17%; n = 14; p = 0.38).

2.3.4. CPCCOEt effect on climbing fibre response is not mediated by mGlu receptors

To test whether the enhancement of the climbing fibre response by CPCCOEt is mediated by $mGlu_1$ receptor, another $mGlu_1$ antagonist was applied while recording climbing fibre responses. After a baseline recording, a competitive $mGlu_1$ antagonist, LY367385 was applied at a concentration that is known to block synaptically evoked $mGlu_1$-EPSPs (100 μM; Miniaci *et al.*, 2001). This on its own did not enhance the climbing fibre response. Furthermore, in the presence of LY367185, CPCCOEt still enhanced the climbing fibre response.

In light of this result it seemed unlikely that $mGlu_1$ was involved but what of other structurally related receptors? Possibilities include other mGlu receptors, especially $mGlu_5$. Also, it has been reported that CPCCOEt has a small negative effect on $mGlu_7$-mediated responses in expressed systems (Litschig *et al.*, 1999). In order to test the involvement of mGlu receptors in the CPCCOEt action on climbing fibre responses, the group I/II mGlu antagonist MCPG (1 mM) or a general antagonist of mGlu receptors (mGlu 1-8) LY341495 (100 μM) was applied while recording the climbing fibre response. However, neither MCPG nor LY341495 affected the climbing fibre response and CPCCOEt still reversibly enhanced it (Figure 2.6). Another family 3 GPCR (therefore a structurally similar receptor), the $GABA_B$ receptor, is also present in Purkinje cells (Hermans and Challis, 2001). To test

whether the non-specific action of CPCCOEt might be mediated by $GABA_B$ receptors, a $GABA_B$ antagonist, CGP55485 (10 μM), was applied while recording climbing fibre responses. In the presence of a GABAb receptor antagonist, CGP55845, applications of CPCCOEt still led to the reversible enhancement of complex spikes. The number of spikelets observed during each complex spike increased in all cells tested (n = 3; Figureure) during CPCCOEt application (Figure 2.7).

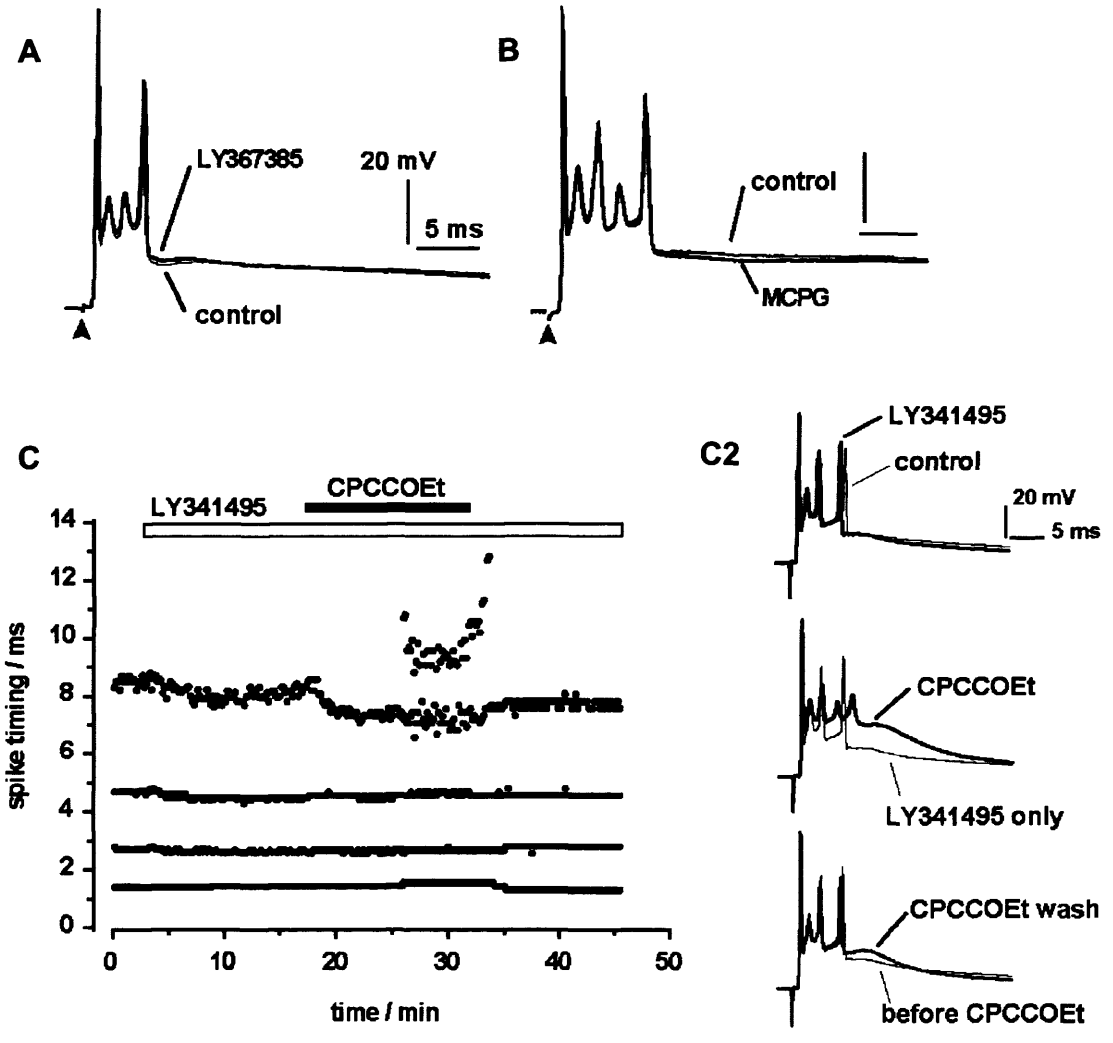


Figure 2.6. CPCCOEt effect on climbing fibre response is not mediated by mGlu receptors. (A) and (B) Examples of climbing fibre responses before and after application of mGlu antagonists. (C1) spikelet occurrences during application of broad mGlu antagonist LY341495 (hollow bar) and when CPCCOEt was additionally applied (filled bar). (C2) Examples of climbing fibre responses during each condition as indicated.

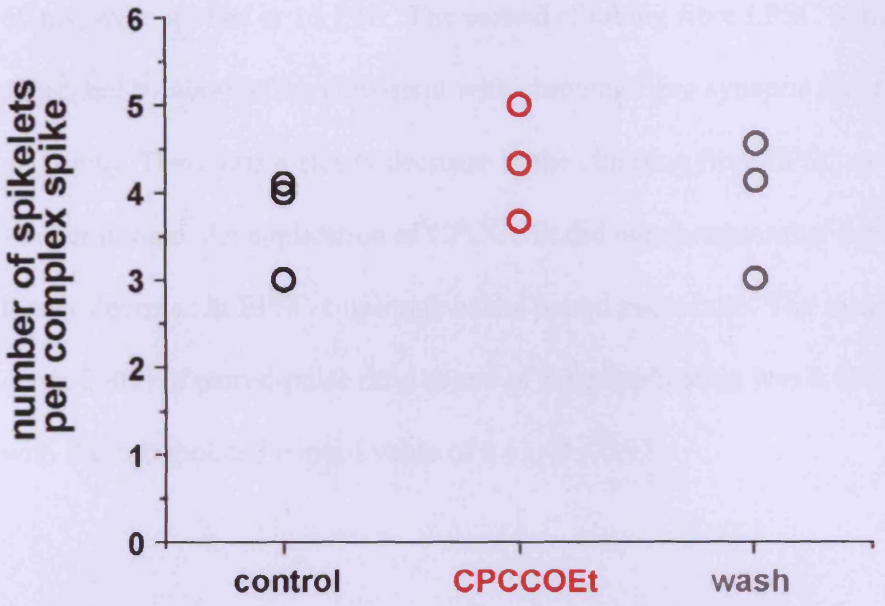


Figure 2.7. CPCCOEt enhances the climbing fibre responses in the presence of CGP55845. The number of spikelets during each climbing fibre response was counted and averaged for a particular cell for each condition. There was a reversible increase in the number of spikelets when CPCCOEt (100 mM) was applied

2.3.5. Does CPCCOEt affect fast glutamatergic transmission?

In order to investigate whether the CPCCOEt enhancement of the climbing fibre responses was of pre- or postsynaptic origin, we voltage-clamped Purkinje cells and recorded the climbing fibre EPSCs.

The enormous dendritic tree of an adult Purkinje cell and the large amplitude of the climbing fibre EPSC makes an adequate space clamp very difficult to achieve. We therefore performed these experiments in the presence of a submaximal concentration of the AMPA receptor blocker NBQX (300 nM). This reduced the amplitude of the AMPA receptor-mediated EPSC such that the cell was unable to escape the voltage-clamp and fire unclamped action potentials (Figure 2.8). Paired stimuli, separated by

60 ms, were applied at 16.7 Hz. The second climbing fibre EPSC of the pair was depressed by about 40%, consistent with climbing fibre synaptic input (Konnerth *et al.*, 1990). There was a steady decrease in the climbing fibre EPSC over time of about 1% per minute. An application of CPCCOEt did not change either the rate of this steady decrease in EPSC amplitude or the paired pulse ratio. The mean value (Neale *et al.*, 2001) of paired-pulse ratio at end of drug application was 0.63 ± 0.02 compared with the extrapolated control value of 0.63; $P = 0.92$.

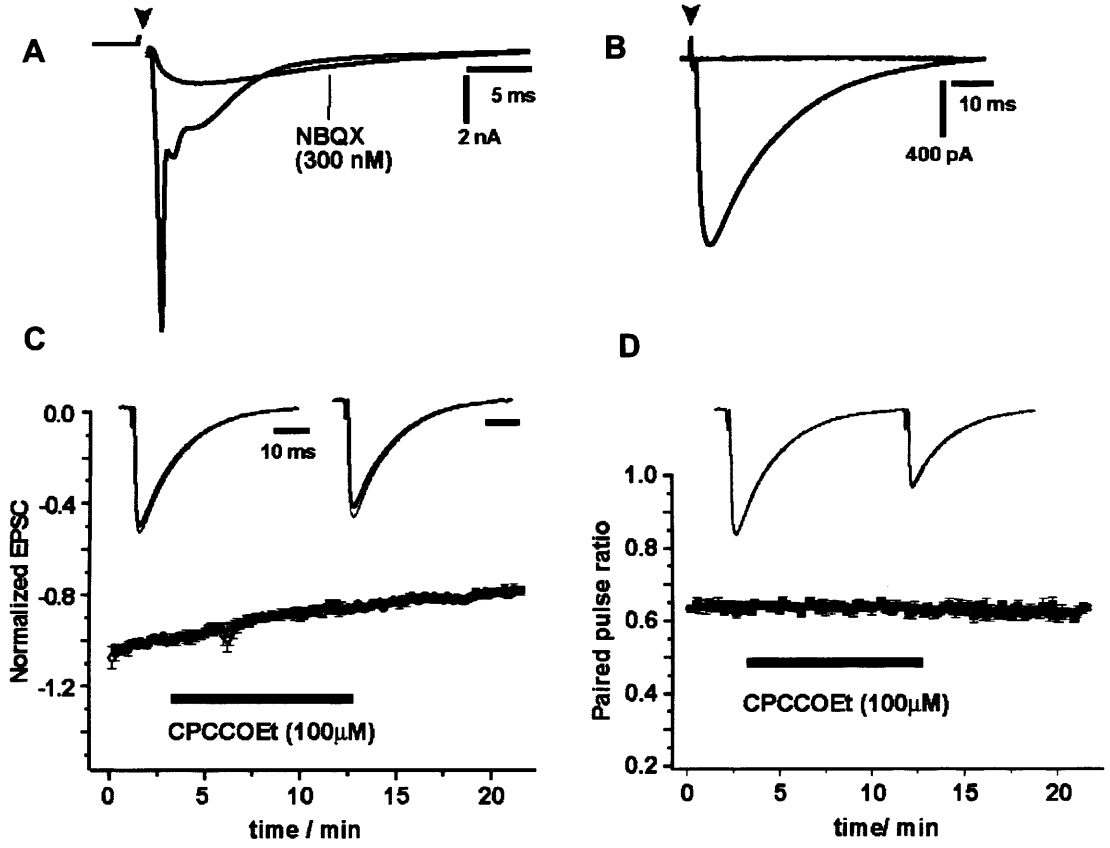


Figure 2.8. CPCCOEt does not affect the climbing fibre EPSC. (A) NBQX (0.3 μM) reduced the amplitude of the climbing fibre EPSC (B) all-or-none cf EPSC evoked with near-threshold stimulation (C) The peak amplitude of the climbing fibre EPSC was normalised against that of the control period. CPCCOEt application did not change the size of the climbing fibre EPSC. (D) CPCCOEt did not affect the paired pulse ratio (EPSC2/EPSC1). N = 3.

2.3.6. Ca^{2+}-activated conductance does not underlie the CPCCOEt effect

Among numerous mechanisms activated by raised [Ca^{2+}] following large Ca^{2+} influx into the dendrites, of particular relevance here is the activation of Ca^{2+} activated potassium channels (K_{Ca}), which are suggested to have a crucial role in terminating the bursting phase (Llinas & Sugimori, 1980a). Big conductance (BK) and small conductance (SK) K_{Ca} channels, in particular, are thought to be activated during bursting (Swensen & Bean, 2003).

In order to investigate if the non-specific action of CPCCOEt is mediated by Ca^{2+}-activated mechanisms, recordings were made with an intracellular solution that contained the fast Ca^{2+} chaelator, BAPTA (40 mM). The experiment was not started until at least 25 minutes after breaking through the membrane under the recording pipette, to allow ample time for BAPTA to reach the Purkinje cell dendrites. Additionally, in some experiments (3 cells), antagonists of SK & BK channels, bicuculline methochloride (100 μM) and iberiotoxin (10 nM), respectively, were added to the external solution approximately 10 minutes before the experiments began. Responses of Purkinje cells to depolarising current injections were recorded in current-clamp at regular intervals during this 10-minute period, to monitor consequences. Purkinje cells responded to depolarising steps with high frequency, small amplitude sodium spikes with much attenuated fast AHP, consistent with previously reported effects of blocking SK and BK channels, respectively (Edgerton & Reinhart, 2003; Llinas & Sugimori, 1980). Baseline climbing fibre responses were then recorded for 10 minutes. However, an application of CPCCOEt (100 μM) still enhanced the spiking phase of climbing fibre responses (Figure 2.9).

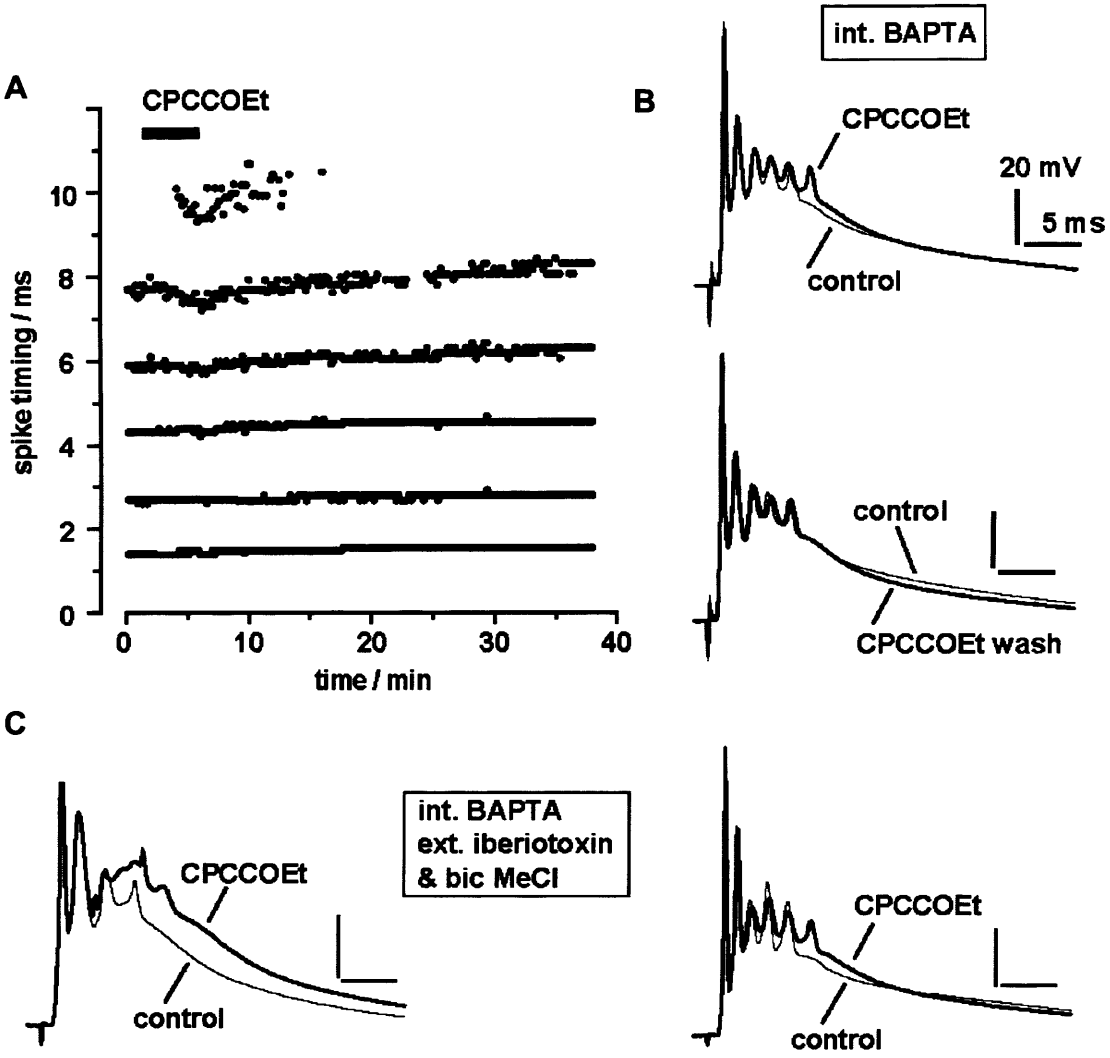


Figure 2.9. The CPCCOEt effect on climbing fibre response is not mediated by calcium-activated K channels. (A) Spike-timing plot of climbing fibre responses recorded from a Purkinje cell filled with BAPTA. CPCCOEt application (filled bar) produced an additional spikelet. (B) Above: examples of climbing fibres in the presence of CPCCOEt (thick line) compared with the control condition (thin line). Below: on washout (thick line), the responses were comparable to the control condition (thin line). (C) Similar results were obtained from two other Purkinje cells filled with BAPTA in addition to a bath application of iberiotoxin and bicuculline methochloride to block BK and SK channels, respectively.

2.3.7. Hyperpolarisation-activated current I_h does not mediate the CPCCOEt effect

I_h in Purkinje cells contributes to setting the membrane potential and resistance at rest (Pape, 1996). I_h is a mixed cation current and is activated by hyperpolarizing potentials and thus contributes to the spontaneous activity of the neuron by providing a depolarising drive to the resting membrane potential. At the membrane potential held (~ -70 mV) in the experiments, I_h is tonically active. I_h is slow to deactivate upon depolarisation relative to the time-course of action potentials. The voltage-dependent time constant for activation and inactivation of I_h is of the order of a few hundred milliseconds (reviewed in Pape, 1996), whereas the spiking phase of a climbing fibre response lasts for approximately 10 ms. It is however possible that some amount of I_h undergoes inactivation during the bursting phase, and this may be modulated by CPCCOEt (Swensen and Bean, 2003). Alternatively I_h may contribute to the waveform of the climbing fibre response by setting the resistance of the membrane.

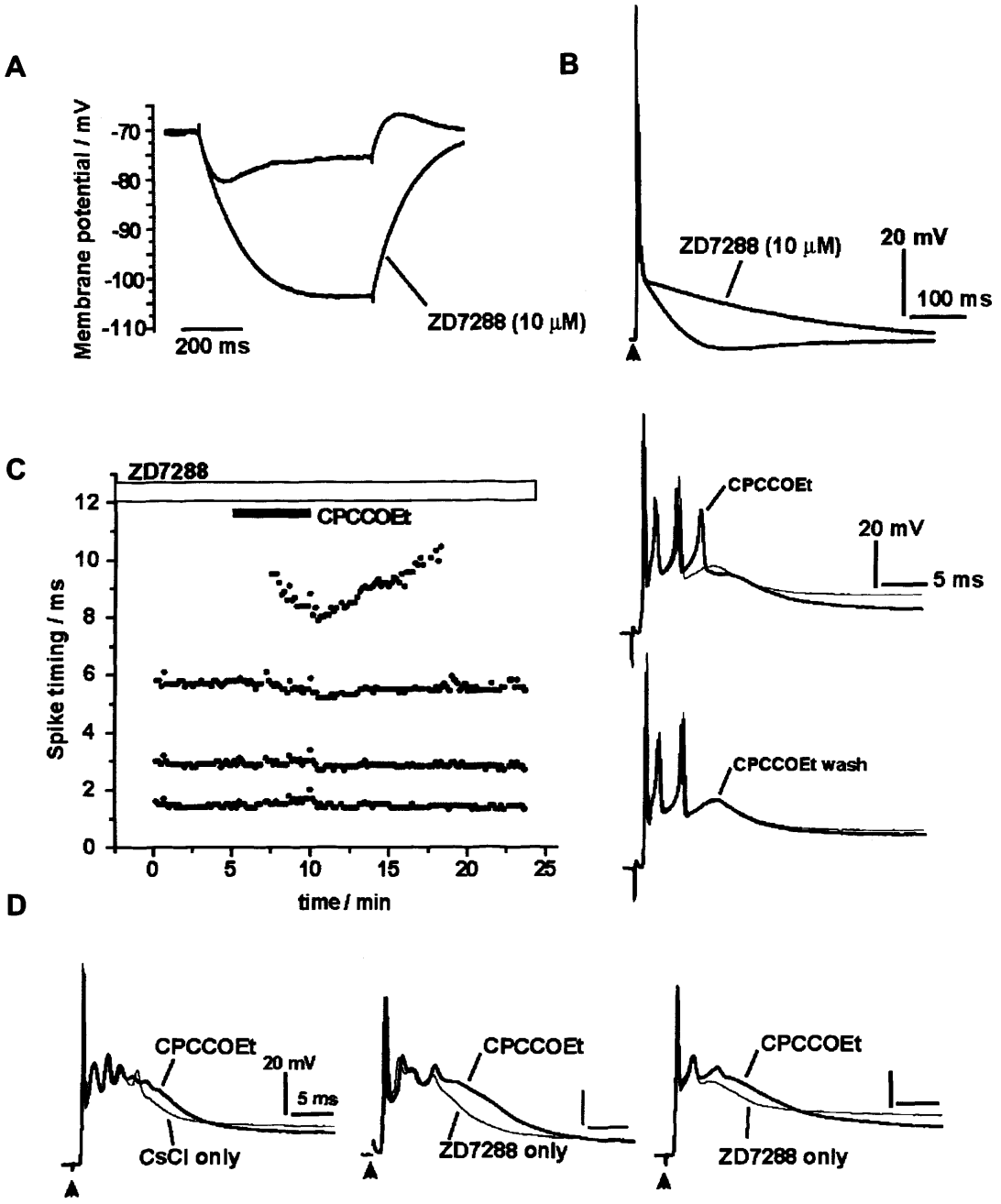


Figure 2.10. CPCCOEt effect is not mediated by I_h. (A) Membrane potential in response to a rectangular hyperpolarizing current injection in the presence of an I_h blocker, ZD7288, compared to control conditions. (B) Climbing fibre response recorded with and without ZD7288 (C) CPCCOEt still produced a reversible enhancement of the spiking phase, as seen in the spike-timing plots and example traces. (D) More examples of CPCCOEt producing an enhancement of the climbing fibre responses, recorded from three separate cells, in the presence of CsCl or ZD7288.

In order to investigate whether the CPCCOEt effect on climbing fibre responses is mediated by I_h, the channel was pharmacologically blocked. A blocker of I_h, namely, ZD7288 (10 μM) or CsCl (1 mM), was applied to the bathing solution while recording climbing fibre responses. This resulted in hyperpolarisation of the cell from a resting membrane potential of -55 mV to approximately -75 mV. Both treatments prolonged the slow phase of the climbing fibre responses, but the spiking phase was not particularly enhanced (Figure 2.10). Furthermore, an additional application of CPCCOEt still produced the enhancement (Figure 2.10).

2.4. Discussion

CPCCOEt effectively blocked $mGlu_1$-mediated responses in rabbit Purkinje cells, with an IC_{50} for the inhibition of the rabbit $mGlu_1$-EPSP of 12.1 ± 8.8 μM. This value is within the range of values reported for rat and human $mGlu_1$ (23 μM for rat mGlu1a (Annoura *et al.*, 1996); 17.4 μM for human $mGlu_{1a}$ (Hermans *et al.*, 1998); 9.9 μM for human $mGlu_{1b}$ (Casabona *et al.*, 1997); and 6.5 μM, for $hmGlu_{1b}$ (Litschig *et al.*, 1999)). This suggests a highly conserved CPCCOEt binding site for rabbit $mGlu_1$, at least for those amino acids crucially involved in CPCCOEt binding.

Further characterisation of CPCCOEt using rabbit cerebellar slices revealed a previously unreported enhancement of climbing fibre responses in Purkinje cells. We then showed that a similar effect occurred in rat Purkinje cells. The lack of climbing fibre response enhancement by other selective $mGlu_1$ or broad-spectrum mGlu antagonists suggests that this is a non-specific action of CPCCOEt. However, all of the other antagonists with activity at $mGlu_1$ receptors that we used were competitive. Agonist-independent activity has not been reported for CPCCOEt, but an inverse

agonist, BAY36-7620, which interacts with a similar region in the transmembrane domain (Carroll *et al.*, 2001;Lavreysen *et al.*, 2003), is reported to inhibit agonist-independent $mGlu_{1a}$ activities (Ango *et al.*, 2001). Therefore there remains the possibility that a non-competitive antagonist such as CPCCOEt affects signalling pathways via the $mGlu_1$ receptor protein in an agonist-independent manner. This now seems less likely in light of the inability of two new non-competitive $mGlu_1$ antagonists to mimic the CPCCOEt effect on the climbing fibre response (see chapter 3).

In the absence of evidence for the involvement of mGlu receptors in the CPCCOEt enhancement we sought further to determine the target. Voltage-clamp experiments indicated that CPCCOEt does not affect the climbing fibre EPSC, narrowing the target mechanism to downstream, voltage-dependent, postsynaptic targets. The multi-component climbing fibre response contains many conductances (Schmolesky *et al.*, 2002) including those directly activated by the voltage changes (Na^+, Ca^{2+} and K^+) or secondary to voltage-gated ion flux (e.g. Ca^{2+}-activated currents). Many of these are difficult to test because of their crucial involvement in synaptic transmission. Additionally, voltage-dependent currents are difficult to study in the large dendritic tree of adult Purkinje cells because of space-clamp considerations. We chose to study two conductance types, Ca^{2+}-activated conductances and the hyperpolarisation-activated non-selective cation conductance, I_h. There are four reasons for examining these particular conductances: (1) both of these conductance types are prevalent in Purkinje cells; (2) they are likely to contribute towards the tail-end of the climbing fibre response where CPCCOEt is seen to act; (3) effective pharmacological tools

exist to test their involvement and (4) their inhibition is not likely to lead to major presynaptic changes affecting transmitter release.

It is suggested that the large influx of Ca^{2+} that occurs during the climbing fibre response activates a number of Ca^{2+}-activated potassium or chloride conductances (Schmolesky *et al.*, 2002). However, because CPCCOEt still enhanced climbing fibre responses, even with 40 mM BAPTA in the internal solution, the action of CPCCOEt on these or any other Ca^{2+}-activated responses is excluded.

Blockade of I_h prolonged the climbing fibre response, with minimal effect on the spiking phase (Figure 2.10). This is consistent with investigations of burst mechanisms in dissociated Purkinje cells (Swensen & Bean, 2003). The prolongation of the repolarising phase is likely to be due to a substantial increase in the input resistance, as it can be seen in the response to hyperpolarising step current injections. The difference in the effect they produce on climbing fibre responses, as well as the persistence of CPCCOEt action on the climbing fibre response in the presence of I_h blockers, therefore, indicate that the non-specific action is not mediated by I_h.

There is a report of K_v1 channels involved in the spiking phase of the climbing fibre response and a pharmacological blockade of such K_v channels (MgTX) produce an effect similar to that seen with CPCCOEt (McKay *et al.*, 2005). However, in the above case, MgTX also acted presynaptically, unlike CPCCOEt. Therefore the mechanism of the non-specific action of CPCCOEt remains unknown, and other studies using this compound as a 'clean' $mGlu_1$ antagonist must be treated with caution. For the proposed *in vivo* experiment, an alternative antagonist is necessary, as

control experiments are not possible without knowing the mechanism of CPCCOEt's non-specific action.

Chapter 3

3.1. Introduction

In Chapter 2, it was concluded that CPCCOEt can no longer be regarded as a specific $mGlu_1$ antagonist on Purkinje cells (and potentially elsewhere). Therefore the interpretation of any of the proposed *in vivo* experiments with this compound would be ambiguous. An alternative antagonist with improved specificity was needed. Around this time, a new generation of $mGlu_1$-selective, non-competitive antagonists started to become available, *e.g.* A-841720, BAY36-7620, JNJ16259685, NPS2390, R214127 and YM-298198 (El-Kouhen *et al.*, 2006; Kohara *et al.*, 2005; Lavreysen *et al.*, 2003a; Lavreysen *et al.*, 2004; Carroll *et al.*, 2001; Lavreysen *et al.*, 2003b; Van Wagenen *et al.*, 2000). We chose to characterise YM-298198 and JNJ16259685 because they were available, they lack inverse agonist activities and they are very potent. Both antagonists are also reported to have a high degree of selectivity for $mGlu_1$ over $mGlu_5$ receptors. For JNJ16259685, IC_{50} for responses mediated by rat $mGlu_1$ and $mGlu_{5a}$ are 3.24 nM and 1.31 μM, respectively (Lavreysen et al., 2004). Similarly, for YM-298198, the IC_{50} value for $mGlu_1$ receptors is 16.9 nM while it has no effect at $mGlu_5$ receptors at the highest concentration tested (10 μM) (Kohara *et al.*, 2005).

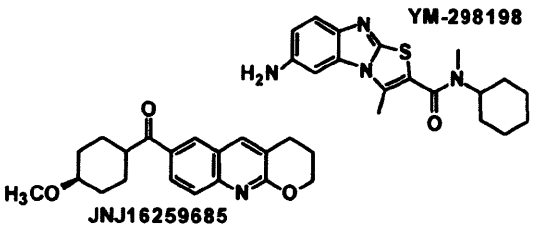


Figure 3.1
Chemical structures of YM-298198 and JNJ16259685

From binding studies, it was suggested that both compounds occupy an overlapping site with CPCCOEt. This is because CPCCOEt displaces YM-298198 from the receptor with a K_i value of 13 μM (Kohara *et al.*, 2005). A shared binding site for JNJ16259685 and CPCCOEt can be inferred from the ability of both JNJ16259685 and CPCCOEt to displace [^{3}H]-R214127 from the $mGlu_1$ receptor. Although these studies are suggestive of a common binding site, they do not necessarily imply overlapping amino acid sequences.

YM-298198 and JNJ16259685 are chemically distinct from CPCCOEt and from each other. However, their specificity needed to be tested, especially on climbing fibre responses. In the previous chapter, a non-specific action of CPCCOEt on the climbing fibre response was concluded from experiments using competitive antagonists only. Here, a lack of effect on climbing fibre responses by YM-298198 and JNJ16259685 needs to be confirmed.

In order to use the antagonists accurately, the time-course of action should be known. Pharmacokinetic analysis of JNJ16259685 into the brain is available. Following subcutaneous injection of 0.63 mg/kg of JNJ16259685 in rats, the level of JNJ16259685 in the cerebellum reached an asymptote in 30-60 min. Only after approximately 2 hours, did the level of JNJ16259685 start to decrease in the brain. The lipophilicity of the antagonist seems to aid in its efficient delivery and persistent availability to the brain. In the proposed behavioural experiments (chapter 4) we intend to deliver the compound locally. Here the time-course of antagonist action is less affected by tissue distribution and penetration of the blood-brain barrier. In a

simplified environment such as that offered *in vitro*, rates of local distribution can be compared with other antagonists.

Therefore the aims of the chapter are to characterise the efficacy, time-course of action and specificity of YM-298198 and JNJ16259685 in cerebellar slices in order to establish their usefulness for *in vivo* behavioural experiments.

3.2. Methods

Methods used in this chapter were the same as in chapter 2 with the exceptions detailed below.

All experiments in this chapter used cerebellar slices from male rats (25-32 days old).

3.2.1. Post-tetanic depression

Single-pulse stimuli were applied to parallel fibres 2 seconds before (EPSP1) and 4 seconds after (EPSP2) a burst stimulation of the same parallel fibres in the presence of AP5 (30 μM), bicuculline (30 μM) and CGP55485 (10 μM). The protocol was repeated every 60 seconds. The burst stimulation consisted of 10 pulses delivered at 100 Hz.

3.2.2. Climbing fibre and parallel fibre responses evoked with single pulses

Climbing fibres and parallel fibres were stimulated in the granule cell layer and molecular layer, respectively. Stimuli for parallel fibres were set so the evoked EPSPs had amplitudes of approximately 6 mV. Stimuli for climbing fibres and parallel fibres were alternated every 30 seconds. A single pulse was used for each stimulus. Because

the presence of post-tetanic depression of parallel fibre synapses was tested in the same cells, the aCSF contained AP5 (30 μM), bicuculline (30 μM) and CGP55485 (10 μM) during recording.

3.2.3. DHPG-induced responses

DHPG-induced responses were measured from Purkinje cells in voltage-clamp. To evoke the response, DHPG (200 μM) was ejected from a glass pipette (same as that used for stimulating parallel and climbing fibres, see chapter 2) onto the dendritic region by a brief (50-500 ms; 3 psi) pressure pulse. The bath solution was aCSF that contained tetrodotoxin (TTX; 1 μM), CsCl (2 mM), bicuculline (30 μM) and NBQX (10 μM) in these experiments.

3.2.4. Pharmacological Reagents

2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), (*S*)-3,5-dihydroxyphenylglycine (DHPG), JNJ16259685 and (*S*)-α-methyl-4-carboxyphenylglycine (MCPG) and CGP55845 were from Tocris Bioscience (Avonmouth, UK). TTX was from Latoxan (Valence, France). YM-298198 hydrochloride was from Ascent Scientific (Weston-Super-Mare, UK).

All pharmacological agents were first prepared as concentrated stock solutions, divided into aliquots and stored as indicated (Table 2.1). Final, working concentrations were obtained by dilution in aCSF (typically at least 1000 fold) immediately before use. In order to stabilise the structure of complex toxin molecules in solution, iberotoxin was dissolved in a 'toxin buffer' that contained NaCl (0.1 M)

and HEPES (20 mM, adjusted to pH 7.4 with NaOH) and aliquots were frozen at -20 °C.

	solvent	concentration	storage temperature / °C
CPCCOEt	DMSO	300 mM	-20
D-APV	dH_2O	100 mM	-20
CGP55845	DMSO	100 mM	-20
bicuculline	DMSO	100 mM	-20
bicuculline methochloride	dH_2O	100 mM	-20
MCPG	1.1 eq NaOH	100 mM	-20
NBQX	1.1 eq NaOH	10 mM	-20
LY341495	1.1 eq NaOH	100 mM	-20
LY367385	1.1 eq NaOH	100 mM	-20
iberiotoxin	toxin buffer	100 µM	-20
ZD7288	dH_2O	100 mM	-20
CsCl	dH_2O	1 M	5
JNJ	DMSO	10 mM	-20
YM-298198	dH_2O	25 mM and 5 mM	-20
YM-298198 hydrochloride	DMSO	25 mM	-20
DHPG	dH_2O	50 mM	-20
TTX	10% ethanoic acid	1 mM	-20

Table 2.1

3.2.5. Data analysis

Data were analysed off-line using Clampfit 9.2 (Molecular Devices) and Origin 6 (Microcal). The peak amplitude of the slow pharmacologically-isolated $mGlu_1$-EPSP was measured relative to the prestimulus baseline. In some cells, the decay phase of the potential that builds up during burst stimulation overlapped with the point where the $mGlu_1$-EPSP amplitude was measured. This was not corrected for.

The DHPG-induced currents were integrated for 600 ms from the time of the pressure application. Climbing fibre responses were quantified by integrating the first 100 ms

of the response (Carta *et al.,* 2006). To measure the extent of short lasting synaptic depression in post-tetanic depression, the ratio of the initial slope (10-60%) of EPSP2 relative to EPSP1 was calculated. Data were normalised to the mean value for the control period (10 minutes). Where appropriate, mean and s.e.m. of the data were obtained and P values calculated by two-tailed t-tests.

3.3. Results

3.3.1. Inhibition of the mGlu$_1$-EPSP by YM-298198 and JNJ16259685

Stable, pharmacologically isolated mGlu$_1$-EPSPs were evoked in Purkinje cells in rat cerebellar slices. Application of JNJ16259685 or YM-298198 resulted in a concentration-dependent reduction in the amplitude of the mGlu$_1$-EPSP (Figure 3.2). IC_{50} values, estimated from sigmoidal fits to the data, were 19 nM and 24 nM for JNJ16259685 and YM-298198, respectively (Figure 3.1). The time-course of inhibition had a slow onset, taking several minutes to reach equilibrium, longer for the lower concentrations. We therefore measured the mGlu$_1$-EPSP remaining after 10 minutes of JNJ16259685 or YM-298198 application (Figure 3.2). We made a direct comparison of the time-course of YM-298198 and MCPG, which from previous studies has shown to exhibit rapid inhibition and reversal on washout. Concentrations close to the IC_{50} value were chosen; YM-298198 (20 nM) and MCPG (100 μM). YM-298198 took approximately 10 minutes to reach equilibrium, whereas the effect of MCPG stabilised within ~3 minutes (Figure 3.2C).

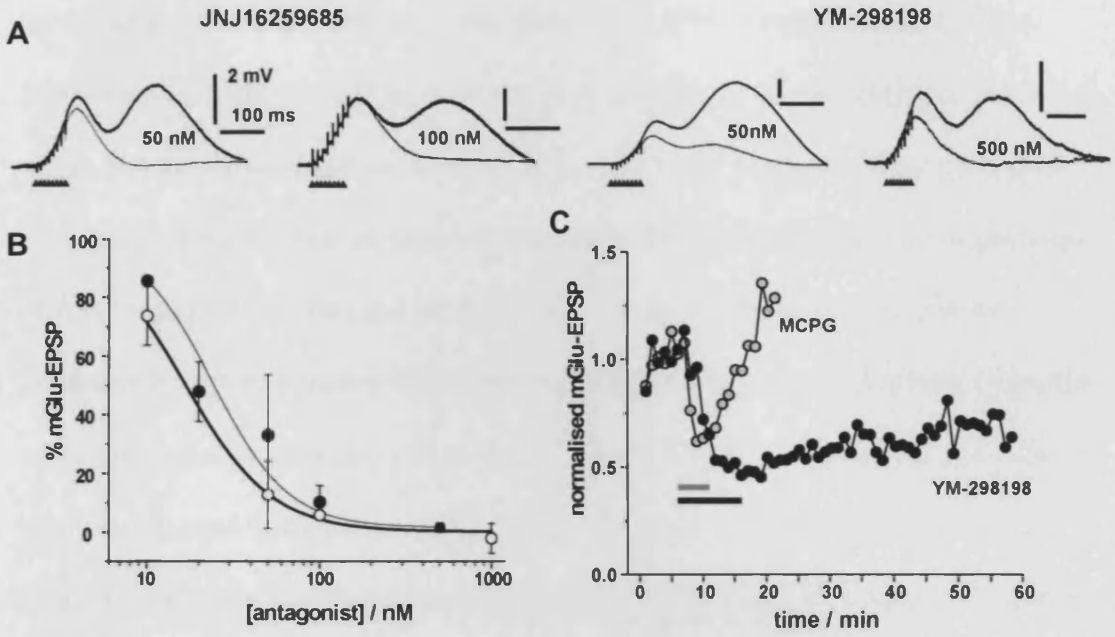


Figure 3.2. JNJ16259685 and YM-298198 potently block the mGlu1-EPSP. (A) Examples showing pharmacologically isolated $mGlu_1$-EPSPs recorded before and at the end of 10-minute application of JNJ16259685 or YM-298198. Concentrations of the antagonists used are indicated. Arrow heads represent times at which parallel fibres were stimulated. (B) Summary of concentration-dependent block of mGlu1-EPSP by JNJ16259685 (hollow circles) and YM-298198 (filled circles). Mean and standard errors of the $mGlu_1$-EPSP remaining after 10 minutes of antagonist application, as a percentage of the control period (n = 3) are shown. Thick and thin lines are the logistic fits to the JNJ16259685 and YM-298198 data, respectively. (c) Time-course action of the effects of two $mGlu_1$ antagonists at concentrations near their IC_{50}. Effects of YM-298198 (20 nM; black bar) and MCPG (100 μM; grey bar) $mGlu_1$ responses from single cells

3.3.2. The nature of the early potentials and their possible sensitivity to YM-298198 and JNJ16259685.

When evoking $mGlu_1$-EPSPs in Purkinje cells, another type of depolarisation was observed, which built up during the burst of parallel fibre stimuli and peaked approximately 120 ms after the first stimulus of the burst. There are two plausible explanations for this 'early potential'. Firstly, it could be due to the electrogenic

uptake of glutamate into Purkinje cells (Otis *et al.*, 1997; Torres-Salazar & Falke, 2007). Or, secondly, it could be due to raised extracellular K^+ concentration following efflux into the extracellular space in the molecular layer. Studies in Bergmann glial cells, where currents were evoked following parallel fibre stimulation in the presence of AMPA and NMDA receptor blockers, indicate that the majority was due to glutamate transporter current while only a small proportion was contributed by raised potassium ion concentrations (Clark & Barbour, 1997). However, in Purkinje cells, it has been reported that treatments that are known to reduce glutamate release from parallel fibre terminals have contrasting effects. Perfusion of Ca^{2+}-free solution results in some reduction of the early potential, while some compounds that are known to act presynaptically to inhibit transmitter release, baclofen and 2-Cl adenosine, do not affect this component (Batchelor & Garthwaite, 1993) while L-AP4 reduces it (Miniaci *et al.*, 2001). It is therefore not clear what proportion glutamate uptake and the extracellular K^+ concentration rise contribute to the early potential in Purkinje cells and this was not investigated in the present study.

In the sample traces presented in Figure 3.2, there is an apparent reduction of the early potential by YM-298198. However, analysis of all cells indicates a high degree of variability in the amplitude of the early potentials during experiments but there was no statistically significant concentration-dependent effect of either YM-298198 (Figure 3.3; $F = 0.80$, $p = 0.55$, one-way ANOVA) or JNJ16259685 ($F = 0.77$, $p = 0.50$, one-way ANOVA, not shown).

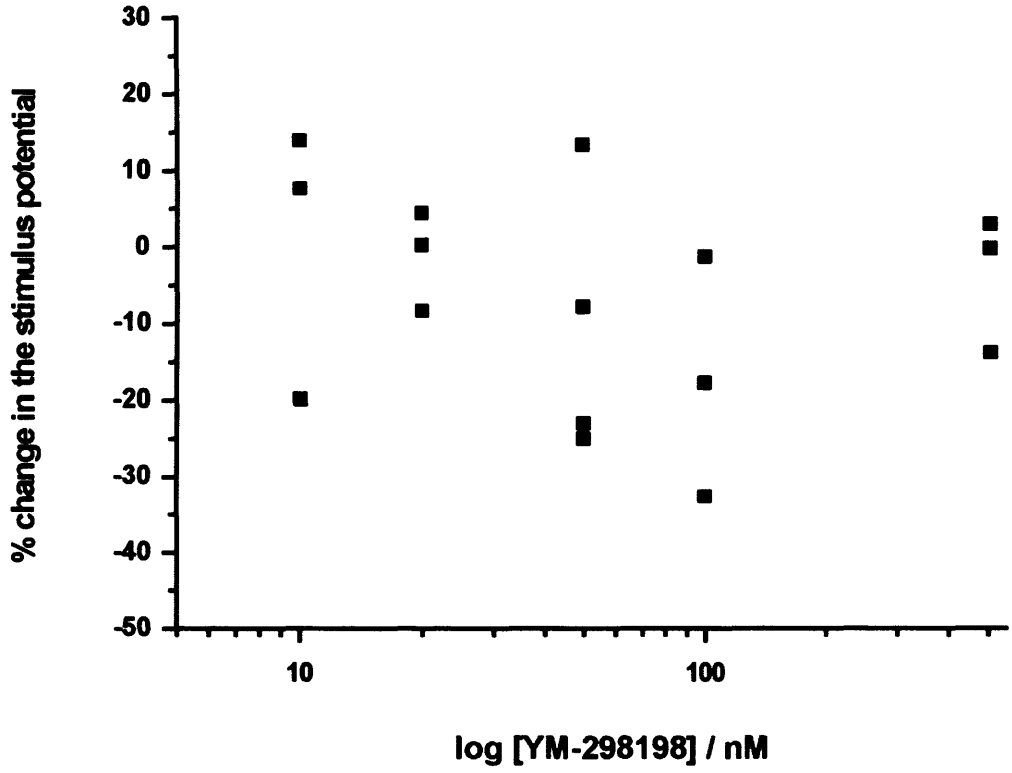


Figure 3.3. YM-298198 does not affect the early potentials. Amplitudes of the early potentials were measured before and at the end of the YM-298198 application for each cell. Changes in the early potential amplitudes were expressed as % of control and plotted against the log of concentration of YM-298198 applied.

3.3.3. Time-course of recovery from YM-298198 and JNJ16259685 actions

Complete reversal of the inhibitory effect of MCPG on the $mGlu_1$-EPSP was achieved within 10 minutes of washing, whereas YM-29818 action had only reversed by ~20% after 40 minutes (Figure 3.2C). However, $mGlu_1$-EPSP is not stable, as evidenced by a gradual decrease in the size of the $mGlu_1$ EPSP that is often seen over tens of minutes of recording. This may be because the protocol used to obtain the mGlu1 EPSP may induce synaptic plasticity. To record $mGlu_1$-mediated responses stably over a long period, the responses were evoked by pressure application of s-DHPG (200 μM). DHPG applied in this manner induced slow, inward currents in recorded Purkinje cells. The response was stable when the inter-stimulus interval was kept

relatively long (90 seconds). After baseline recording for 10 minutes, high concentrations of YM-298198 (1 μM) or JNJ16259685 (500 nM) were applied for 5 minutes. This produced a block of the DHPG-induced inward current within 2 minutes. The perfusion was then switched back to a $mGlu_1$ antagonist-free solution and recovery of the response to DHPG application was monitored. Recovery of the DHPG-induced inward current was slow for YM-298198 compared to that of MCPG (9 min). Half recovery times from YM-298198 and MCPG were 74 minutes and 9 minutes, respectively. No recovery from JNJ16259685 was observed over this time span (Figure 3.4).

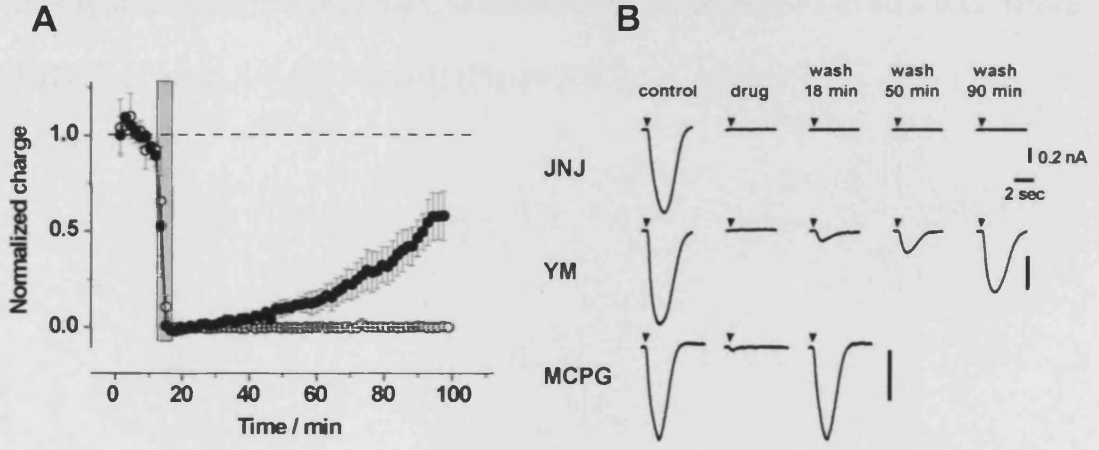


Figure 3.4. Recovery time-course from JNJ16259685 and YM-298198 actions. (A) A plot of DHPG-induced inward current that was integrated and normalised against the first 10 minutes of recording. The grey bar represents the time of JNJ16259685 (500 nM) or YM-298198 (1 μM) application. (B) Examples showing the DHPG-induced current at the times indicated. Downward arrowheads represent the time at which DHPG was applied with pressure. Vertical bars correspond to 0.2 nA.

3.3.4. Specificity of JNJ16259685

The specificity of JNJ16259685 was tested on parallel fibre and climbing fibre responses evoked by single stimuli. Alternating stimuli were applied to parallel fibres and climbing fibres. After baseline recording for 10 minutes, the perfusate was

switched to a solution containing JNJ16259685 (10 μM). This did not change the amplitude or the shape of the parallel fibre responses (Figure 3.5A). The slope of the rising phase of EPSP after application of JNJ16259685 was 107 ± 4% of the control ($P = 0.25$, $n = 3$). Similarly, the area under the climbing fibre response waveform was not affected (99 ± 1%; $P = 0.69$, $n = 3$) and neither was the spiking phase of the complex spike also affected (Figure 3.5B). The presence of JNJ16259685 was tested by the presence of an $mGlu_1$-dependent short-term plasticity in parallel fibre responses, namely post-tetanic depression (Neale et al., 2001). All cells included in the study showed post-tetanic depression before, and impaired post-tetanic depression after application of JNJ16259685, as measured by EPSP2/EPSP1 (0.46 ± 0.12 before; 0.87 ± 0.11 after; $n = 4$; $P = 0.003$) (Figure 3.5C).

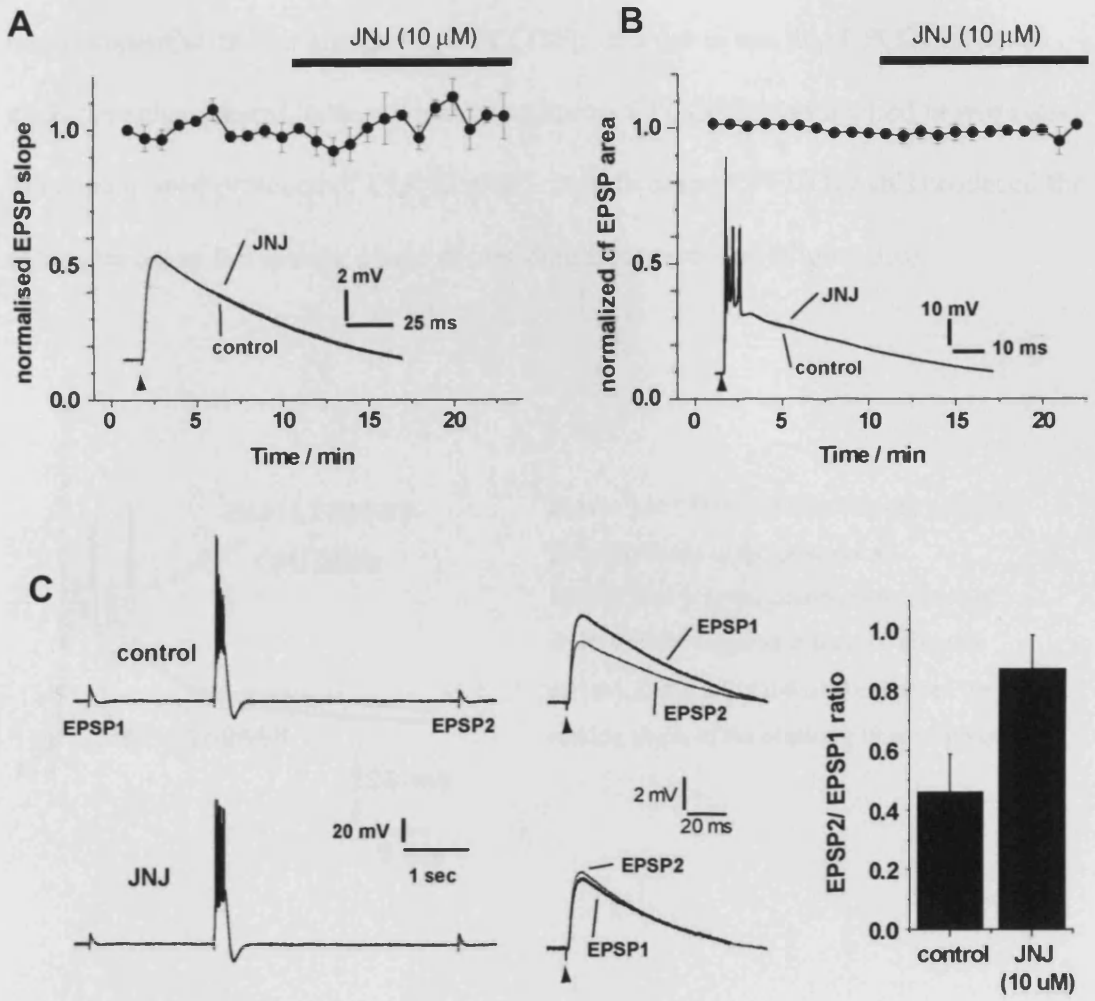


Figure 3.5. JNJ16259685does not affect parallel fibre or climbing fibre responses. (A) The initial rising phase (10-60%) of the parallel fibre EPSP was normalised against that of control period (first 10 min). Means and standard errors are plotted. JNJ16259685 (10 μM) was applied (filled bar). An inset shows examples of pf EPSP recorded during control period and at the end of 10 min drug application. (B) The integral of the climbing fibre response is plotted. Filled bar represents JNJ16259685 application. An inset shows example climbing fibre responses recorded during control period and at the end of 10 min drug application. (C) Post-tetanic depression was observed during control period (top) but not in the presence of JNJ16259685. The recordings were obtained from the same cells as those used in (A) and (B). The bar graph shows that the post-tetanic depression, expressed as EPSP2/EPSP1, is significantly reduced in the presence of JNJ16259685 (n = 4).

JNJ16259685 did not mimic the enhancement we reported, in chapter 2, for another non-competitive $mGlu_1$ antagonist, CPCCOEt. In order to test that CPCCOEt could elicit the enhancement in the present experiments CPCCOEt was applied in two cells in the continued presence of JNJ16259685. In both cases, CPCCOEt still produced the enhancement of the spiking phase of climbing fibre response (Figure 3.6).

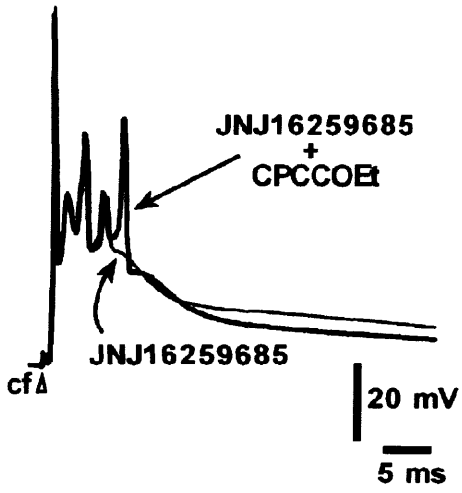


Figure 3.6. CPCCOEt enhances the climbing fibre responses in the presence of JNJ16259685. In the continued presence of JNJ16259685 (applied at least 10 minutes earlier), CPCCOEt (100 μM) enhanced the spiking phase of the climbing fibre response.

3.3.5. Specificity of YM-298198

Similar specificity experiments also tested YM-298198. Briefly, after 10 minutes baseline recording, perfusion was switched to solution containing YM-298198 (10 μM). YM-298198 neither affected pf EPSPs (initial slope was 110 ± 10% of control, $P = 0.32$, $n = 4$) nor climbing fibre responses (102 ± 5% of control, $P = 0.83$, $n = 3$). All cells included in the study showed post-tetanic depression before the YM-298198 application (Figure 3.7C), while PTD was significantly reduced in the presence of YM-298198 (0.56 ± 0.04 before, 0.97 ± 0.05 after, $n = 4$, $P = 0.005$).

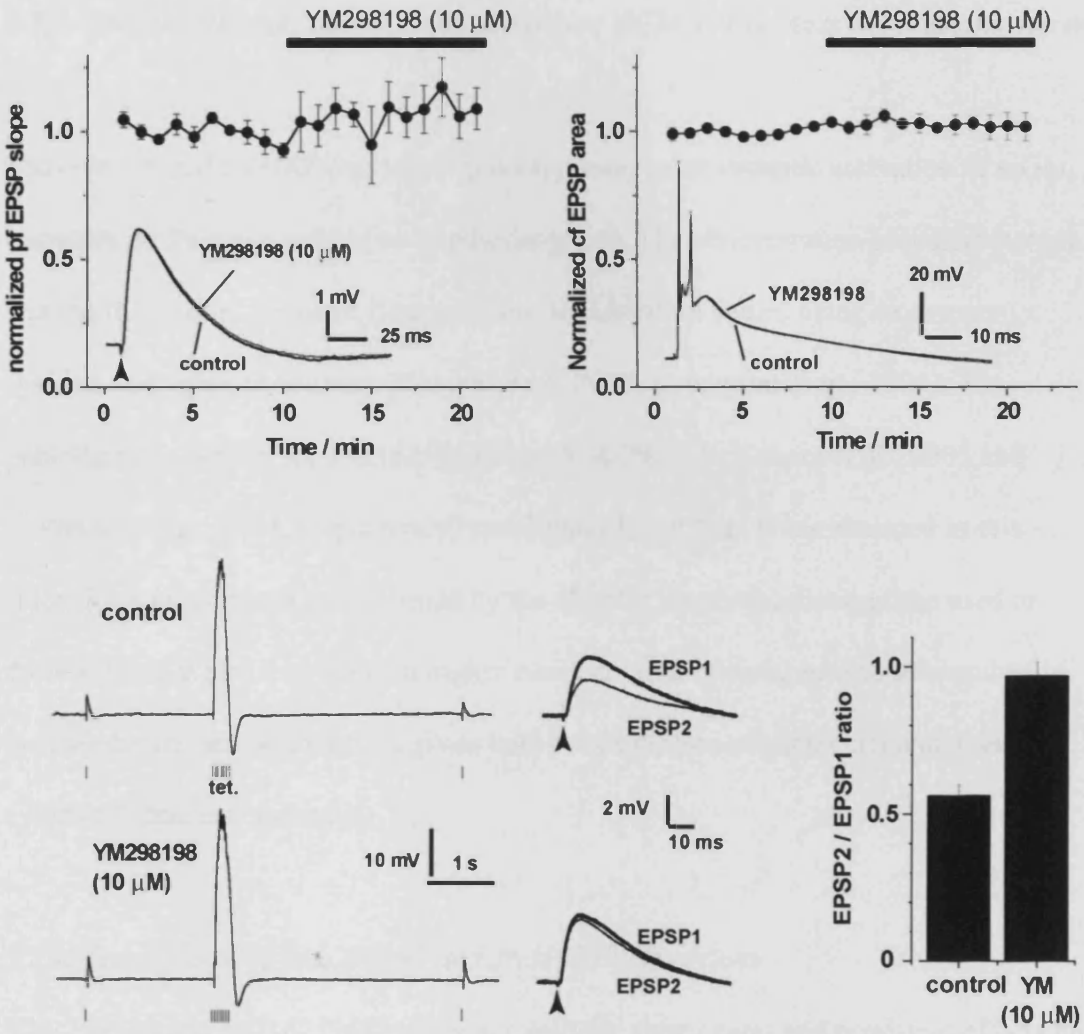


Figure 3.7. YM-298198 does not affect pf- and cf- responses. (A) The initial slope of pf-EPSP over time. Filled bar represents YM-298198 application. Inset shows example pf-EPSPs before and at the end of YM-298198 application. (B) The integral of cf-responses over time. Filled bar represents YM-298198 application. Inset shows example cf responses before and at the end of 10 minute YM-298198 application. (C) Post tetanic depression of pf-EPSP is present before YM-298198 but is significantly reduced in the presence of YM-298198 (n = 4).

3.5. Discussion

3.5.1. YM-298198 and JNJ16259685 are potent $mGlu_1$ antagonists in cerebellar slices

YM-298198 and JNJ16259685 both potently antagonise synaptic activation of $mGlu_1$ receptors on Purkinje cells in rat cerebellar slices. The concentration-response curves and the IC_{50} values obtained here are close to published values using expression systems and cultured neurons (Kohara *et al.*, 2005; Lavreysen *et al.*, 2004). The published IC_{50} values for JNJ16259685 and YM-298198 (Kohara et al., 2005 and Lavreysen et al., 2004, respectively) are slightly lower than those obtained in this study. This may in part be explained by the slightly longer incubation time used in these studies, which may allow a higher concentration of antagonist to accumulate in the membrane (see below) for a given bath concentration of antagonist and, therefore, a higher "apparent" potency.

3.5.2. Time-course of YM-298198 and JNJ16259685 actions

YM-298198 and JNJ16259685 showed relatively slow onsets and persistent effects on $mGlu_1$ receptor mediated responses. Poor reversibility of the inhibition by JNJ16259685 is in agreement with Lavreysen *et al.* (2004) who report substantial occupancy of $mGlu_1$ receptors by JNJ16259685 in CNS for several hours following a subcutaneous administration (Lavreysen *et al.*, 2004). The availability of the compound in this case correlates with the level of JNJ16259685 in the plasma (Lavreysen *et al.*, 2004), so the persistent availability probably reflects a lack of fast metabolism and excretion mechanisms. The slow onset of YM-298198 and JNJ16259685 effects in slice is suggestive of the membrane route of action (Kenakin,

1997). That is, for the antagonist to work, it needs first to diffuse into and accumulate in the membrane, where the interaction with receptor subsequently takes place. This membrane route of action is consistent with the reported interactions of JNJ16259685 and YM-298198 with the transmembrane, rather than the extracellular, domains. In slice, compared to reduced, expression systems, there is probably an additional rate limiting factor introduced by the presence of overlying tissue, rich in lipid that can delay the antagonist action before it can reach the relevant receptors located on the dendrites of the recorded Purkinje cell. Such effects might be expected to similarly slow the action of JNJ16259685 and YM-298198 *in vivo*.

3.5.3. Specificity of YM-298198 and JNJ16259685

YM-298198 and JNJ16259685 appeared specific for $mGlu_1$ receptors and had no non-specific action on the climbing fibre and parallel fibre responses activated by single pulses. Of interest is a lack of effect on the climbing fibre response. In chapter 2, we have shown that the $mGlu_1$ antagonist, CPCCOEt, enhanced the climbing fibre responses. The lack of effect of these newer antagonists is consistent with the conclusion that this action of CPCCOEt was not mediated by $mGlu_1$ receptors.

The finding in this chapter that both JNJ16259685 and YM-298198 did not produce observable effect on the climbing fibre response disagrees with a conclusion drawn by Carta et al. (2006), who concluded that $mGlu_1$ activation during climbing fibre activation enhances the long lasting after-depolarisation. In their study they used a competitive $mGlu_1$ antagonist LY367385, which produced a substantial reduction of the slow depolarisation (up to ~100 ms after the response onset) of the climbing fibre responses (Carta *et al.*, 2006). The authors attributed this effect to a block of climbing

fibre activation of $mGlu_1$ receptors. Here, neither JNJ16259685 nor YM-298198 produced this effect. The difference could be explained by the fact that LY367385 is an inverse agonist (Pula *et al.*, 2004). In the experiments described in this chapter, an application of LY367385 produced a reduction in the membrane resistance. Furthermore, the lack of effect on climbing fibre responses by JNJ16259685 or YM-298198 cannot be explained by a failure of the antagonists to reach a sufficient concentration, because the presence of the antagonists is indicated by an inhibition of PTD, which is dependent on $mGlu_1$ activation (Neale *et al.*, 2001).

3.5.4. Advantages of JNJ16259685 and YM-298198 for use in the clinic and behavioural experiments.

JNJ16259685 and YM-29818 are potent $mGlu_1$ antagonists and selective over other mGlu receptors. They were both found to be persistent in their action. $mGlu_1$ antagonists may have possible uses in anti-inflammatory pain, as reviewed in Gasparini *et al.*, 2002. The lipophilicity of the compounds also makes these compounds attractive for therapeutic use. It allows the drugs to cross the blood-brain barrier, making drug administration easier. The persistence of action also allows infrequent administration.

For the proposed behavioural experiment, the time-course of antagonist action that is of the order of an hour or more is advantageous, as a typical training session lasts for about an hour (chapter 4). The *in vitro* assessments described here suggest both JNJ16259685 and YM-298198 may be suitable for use in the proposed behavioural experiment. However, given the higher potency, there may be an advantage in using JNJ16259685.

Chapter 4

4.1. Introduction

In this chapter, potential relationships between pf-PC LTD and one form of cerebellum-dependent learning are examined. Classical conditioning of the NM response in the rabbit is the target behaviour and the role of cerebellar cortical $mGlu_1$ is investigated. Functions of $mGlu_1$ are assessed first in the acquisition of conditioned responses and second, in the execution of conditioned responses.

4.1.1. Pharmacological inactivation of cerebellar cortical mechanisms in NM conditioning.

Drugs that have been successfully used in the cerebellar cortex to probe its functions include CNQX (AMPA/kainate receptor antagonist; (Attwell *et al.*, 2001; Carter & McElligott, 2005)), and muscimol ($GABA_A$ receptor agonist; Attwell *et al.*, 2002). However, both drugs produce a substantial change in the baseline firing rate of Purkinje cells. Muscimol strongly, if not completely, reduces Purkinje cell spiking via hyperpolarisation. Interestingly, in the cerebellar slice, CNQX does not prevent the generation of Purkinje cell simple spikes, which appear to be maintained by intrinsic mechanisms (Hausser & Clark, 1997). However, *in vivo*, cortical application of CNQX may lead to the reduction or abolition of simple spikes (Wada *et al.*, 2007) because Purkinje cells continue to receive substantially more GABAergic inputs, probably because more of the spontaneously active molecular layer interneurons *in vivo* are intact than *in vitro*. Thus, a consequence of AMPA/kainate receptor block *in vivo* may be to remove the glutamatergic excitatory drive on Purkinje cells while GABAergic inputs to them remain active, with a resultant hyperpolarisation. In

mutant mice with conditional expression of tetanus toxin in granule cells, which prevents glutamate release from the granule cell axons, recordings show loss of simple spike activity in Purkinje cells (Wada *et al.*, 2007).

Thus, studies that have employed pharmacological disruption of cortical mechanisms in NM conditioning have not been capable of identifying specific cellular and molecular mechanisms in learning because they have introduced non-specific changes in firing rates and in the ability of the cortical neurons to code the CS and US-related information normally. Although analyses of post-training mechanisms during a "consolidation" time window has helped dissociate cortical and nuclear mechanisms after training (Attwell *et al.,* 2002), a direct analysis of acquisition mechanisms has been difficult.

4.1.2. Analysis of $mGlu_1$ function in NM conditioning could provide a direct test of acquisition mechanisms

In vitro experiments have revealed that $mGlu_1$ activation is required for the induction of pf-PC LTD, but not for its maintenance. For example, application of AIDA, an $mGlu_1$ antagonist during, but not after, conjunctive pf and cf stimulation, leads to an impairment of pf-PC LTD induction (Karachot *et al.*, 2001). If this conventional pf-PC LTD provides an exact match to the processes underlying cerebellum-dependent associative learning, then $mGlu_1$ block during acquisition of conditioned responses should impair learning but a block after acquisition should not affect retention of the conditioning.

The effect of $mGlu_1$ antagonist on the baseline activity of Purkinje cells *in vivo* is not known. However, in slices, $mGlu_1$ antagonists do not affect spontaneous firing or rapid cf- and pf- EPSPs in Purkinje cells (Fukunaga *et al.*, 2007). Because of its lack of effects on AMPA, NMDA and $GABA_B$ receptors, the use of the $mGlu_1$ antagonist JNJ16259685 *in vivo* for an analysis of cortical mechanisms in NM conditioning is likely to have substantial advantages over previous studies. This $mGlu_1$ antagonist is unlikely to have a significant effect on the baseline firing rate of Purkinje cells and consequently that of the target neurons in the DCN, though this proposition will need experimental validation. Thus, it is unlikely that JNJ16259685 will cause a major change in spike modulation in response to CS and US presentations. If the $mGlu_1$ antagonist were to have a significant effect on the acquisition of NM conditioning and baseline firing rate changes could be excluded, then this would be a very direct test of the relationship between pf-PC LTD mechanisms and behavioural learning.

4.1.3. $mGlu_1$ receptor function and online control of Purkinje cell activity

As introduced in Chapter 1, some models suggest that the signalling cascade which follows $mGlu_1$ receptor activation is directly responsible for generating learned, well-timed pauses in Purkinje cell simple spikes that are proposed to occur in response to CS-related parallel fibre activity, and which have been seen in the decerebrate preparation (Jirenhed and Hesslow, 2006). If these models as postulated by Fiala *et al.* (1996) and Steuber *et al* (2004) are correct, then it becomes important to test the effects of $mGlu_1$ antagonist on the execution of conditioned responses. The models predict that there should be significant effects on the topography of previously acquired conditioned responses or even that they might be abolished.

The behavioural experiments described in this chapter will, therefore, test the effects of the $mGlu_1$ antagonist, JNJ16259685, in the eyeblink zone of cerebellar cortex on the execution, as well as the acquisition, of conditioned NM responses.

4.2. Methods

4.2.1. Pre-operative treatments

Male New Zealand White rabbits (2.5-3.0 kg) were used. Subjects were anaesthetised with fentanyl-fluoanisone (Hypnorm; 0.05-3.3 mg/kg i.m.) and benzodiazepine (valium; 0.25 mg/kg, i.v.). Enrofloxacin (Baytril; 5 mg/kg, s.c.) and meloxican (metacam; 0.3 mg/kg, s.c.) were administered to control infection and inflammatory pain, respectively, during and after the surgery. Each subject was intubated with a lubricated endotracheal tube (3.5 mm o.d.) and given an intravenous infusion of an osmotic diuretic agent mannitol (20% w/v, 25 ml over 10 min) 40 minutes before the surgery to decrease the intracranial pressure.

4.2.2. Surgery

Subjects were maintained under gaseous anaesthesia (isofluorane in N_2O and O_2 mixture (40:60); 5% for induction and 1.5-2% for maintenance). An incision was made in the skin and the tissue underneath was retracted to expose the skull. A small craniotomy (~1.0 cm in diameter) was made with dental drill and a guide cannula (26 ga) was lowered into the right cerebellar lobule HVI with visual guidance (Figure 4.1), to the depth of 2.5 mm below brain surface. The guide cannula was fixed with dental acrylic, and a dummy stylus was placed in the bore. The skin was sutured and antibiotic powder dusted around the wound. Buprenorphine (30 μg/kg i.m.) was

administered for post-operative analgesia. Gas anaesthesia was withdrawn and each subject was allowed to recover in a quiet, warm environment and then returned to the home cage.

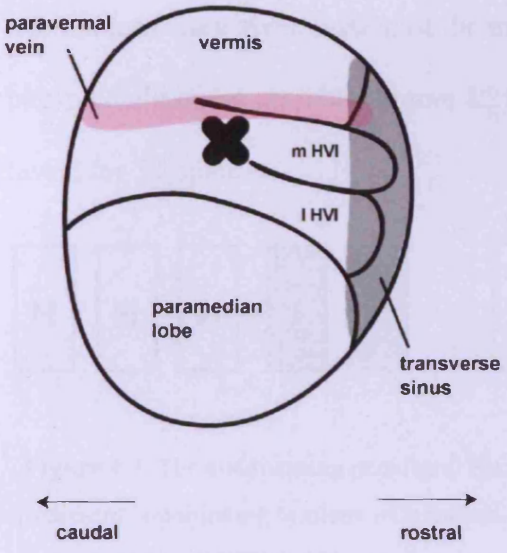


Figure 4.1. A typical view through the cranial opening, where the medial and lateral lobules of HVI (m HVI and l HVI, respectively) can be seen between the vermis and the paramedian lobe. The target entry point for the guide cannula is marked with a cross.

All surgical instruments, implanted cannulae, and drapes had been sterilised in an autoclave (135 ºC for 10 minutes). Additionally, chlorhexidine solution (10% in 95% ethanol) was applied on surfaces for further sterilization, where appropriate.

4.2.3. Post-operative treatments

All subjects were given antibiotics (enfrofloxacin, 5 mg/kg) and Buprenorphine (30 μg/kg i.m.) for 3 days.

4.2.4. Conditioning paradigm

There was a one-week post-operative recovery period for each subject and then the conditioning protocols began.

Short-protocol: the following conditioning design was designed to achieve asymptotic learning within 2 training sessions, and was inspired by an earlier study from the laboratory, where it was observed that subjects learned more quickly with greater number of habituation sessions (Hardiman *et al.*, 1996). Here, 3 sessions of habituations were given instead of the more usual single session, before subjects began conditioning sessions (Figure 4.2). Habituation and conditioning sessions each lasted for 50 minutes.

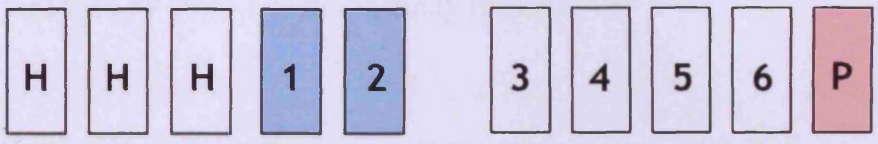


Figure 4.2. The conditioning paradigm. Each box represents a session. Coloured boxes represent conditioning sessions with infusions. H = habituation session. P = performance test with CNQX or JNJ16259685,

Subjects were placed in custom-made restraining stocks. On the right side, a nylon loop was sutured in the nictitating membrane under local anaesthetic (proxymetacain) to allow an isotonic transducer to be attached and to record its movement. Two stainless-steel wound clips were applied to the periocular skin for delivering unconditional stimuli. Subjects were removed from the restraining stocks and allowed to rest for 3 hours.

Habituation sessions: For the habituation sessions, subjects were placed in the restraining stocks, the NM response transducers and US delivery leads were attached and they were placed in ventilated, sound attenuating chambers (conditioning chambers). No CS or US was delivered during the habituation session. The

background noise from the ventilation fan was 59 dB. The subjects habituated to these conditions for three sessions (50 minutes each, once a day).

Conditioning sessions: Each conditioning session consisted of 100 trials. The CS was a 410 ms duration, 1 kHz tone presented from speakers inside the conditioning chamber at 85 dB. The US was a 2 mA current for 60 ms (6 x 10 ms pulses, bipolar), 350 ms after the onset of the tone. Every 10^{th} trial was a CS-only trial. Trials were separated by 30 ±5 s semi-randomly selected.

Sessions 1-2: Conditioning sessions with infusions. An infusion was made in each subject 15 minutes before the start of each session. A 33 ga injector was inserted down the implanted guide cannula to protrude 1.5 mm below the end of the guide. 2 µl infusion of vehicle (0.1% DMSO), JNJ16259685 (10 µM or 1 mM with 0.1% or 1% DMSO, respectively) or CNQX disodium salt (5 mM) in PBS, was made over 2 minutes by means of a 10 µl Hamilton syringe connected to the infusion cannula with fine polythene tubing.

Group	First 2 conditioning sessions	Number of subjects
Vehicle control	0.1% DMSO	5
$mGlu_1$ antagonist	JNJ16259685 (10 µM)	5

CNQX	CNQX (5 mM)	5
$mGlu_1$ antagonist	JNJ16259685 (1 mM)	2
$mGlu_1$ antagonist	YM-298198 (200 μM)	1

Table 4.1. Groups used in the study

Sessions 3-6: conditioning sessions without infusions. All subjects were conditioned for further 4 sessions without infusions to test that they were able to acquire conditioned responses.

Session P: CNQX performance test. After baseline (20 trials), CNQX (2.5 mM in 0.1 M phosphate buffered saline) was infused down the existing guide cannula. A smaller dose of CNQX was given here because it gives a more sensitive measure of the proximity of cannula tips to the critical region, whereas the aim of CNQX infusion during the acquisition session was to ensure as large an effect as possible. To provide an estimate of the location of the guide cannula, by measuring the time for the drug to take effect and to test for the magnitude of the behavioural effects, the conditioning session was started immediately after infusion.

Session Pii: JNJ16259685 performance test. An additional session performance test was carried out in some subjects, where JNJ16259685 (10 μM) was infused to test the effects of $mGlu_1$ receptor activation on CR execution.

4.2.5. Data acquisition and analysis

Movement of the NM was measured by an isotonic transducer and the signal was fed into an analogue to digital converter (CED Micro 1401). Baseline and within-trial position of the NM was acquired using custom software, (Blink, Peter Trigg UCL). Conditioned responses were defined by a closure of nictitating membrane with onset of displacement >35 ms after the onset of the CS, and with amplitude > 0.5 mm. Trials during which movement of nictitating membrane occurred during the baseline period were not included in the analyses. The acquisition and analysis of the data was with custom software, (Blink, Peter Trigg, UCL).

To compare the learning rates between groups, the numbers of trials taken to reach 50% CR level of the subjects were compared using one way ANOVA (SPSS). Where there was between-group interaction, *post-hoc* Student-Newman-Keuls test was used to see the origin of the difference. To assess the effect of JNJ16259685 infusions on the profile of conditioned responses, peak amplitude, onset latency and peak latency of the conditioned responses were analysed using repeated measures ANOVA (Sigmastat).

4.2.6. Histology

After the behavioural experiment, subjects received heparin (i.v.) followed by an overdose of pentobarbitone (Euthatal; i.v.), transcardiac perfusion of 0.9% saline, and then formalin solution (4% w/v). The procedure for the perfusion is similar to that described in Chapter2. After perfusion, the skull was opened and the brain was removed. The whole brain was fixed in formalin solution (20% sucrose, 4% formaldehyde) for 2 days before embedding in gelatin. For embedding, the formalin

was washed from the brain by soaking in fresh tap water for 4 hours and the overlying arachnoid matter and blood vessels were removed under a dissection microscope. The brain was submerged in 5%, followed by 10% gelatin solution, for 30 min each at 50 °C under reduced pressure. The gelatin block was then allowed to set, fixed in formalin solution (4% formaldehyde, 20% sucrose), and then the embedded tissue was frozen and serially-sectioned on a sledge microtome. 50 μm-thick sections were cut and mounted on gelatine-subbed glass slides. The sections were air dried overnight. Every 4^{th} section was stained with Nissl stain, dehydrated in an alcohol series and mounted under a glass cover slip with a plastic mountant.

4.3. Results

4.3.1. Acquisition of conditioning during block of cortical $mGlu_1$ receptor function

After three habituation sessions, animals received two conditioning sessions with cortical infusions. The learning curves of the JNJ16259685 Group and the Vehicle Control Group overlapped closely (Figure 4.3a). There was no significant difference in the number of trials required to reach 50% CR level (140.8 ± 11.7 and 144.6 ± 15.4 trials for vehicle and JNJ16259685 groups, respectively; Figure 4.3b). In contrast, no appreciable level of CRs was detected during sessions 1 & 2, in the CNQX Group subjects. For the CNQX Group, CR frequency at the beginning (during the first 10 trials) of session 3 was 2 ± 2 %, compared to 88 ± 12% and 93.3 ± 3.3% in the JNJ16259685 and Vehicle Control groups. However, in the third session without CNQX infusion, these subjects acquired CRs at a rate comparable with that of other subjects in session 2, although the onset of learning after the removal of CNQX was slightly earlier than onset of learning expected from a naïve state i.e. as compared to the learning curves of the vehicle control and JNJ16259685 groups. The mean number of trials taken by the subjects in CNQX Group was 265 ± 12 (n = 5). This is significantly different from those of the Vehicle Control Group and the JNJ Group. One way ANOVA showed a significant between-group difference ($F = 26.5$, $p<0.01$), arising from the slow learning rate of CNQX Group compared to the Vehicle Control and JNJ Groups (Student-Newman-Keuls *post hoc* test).

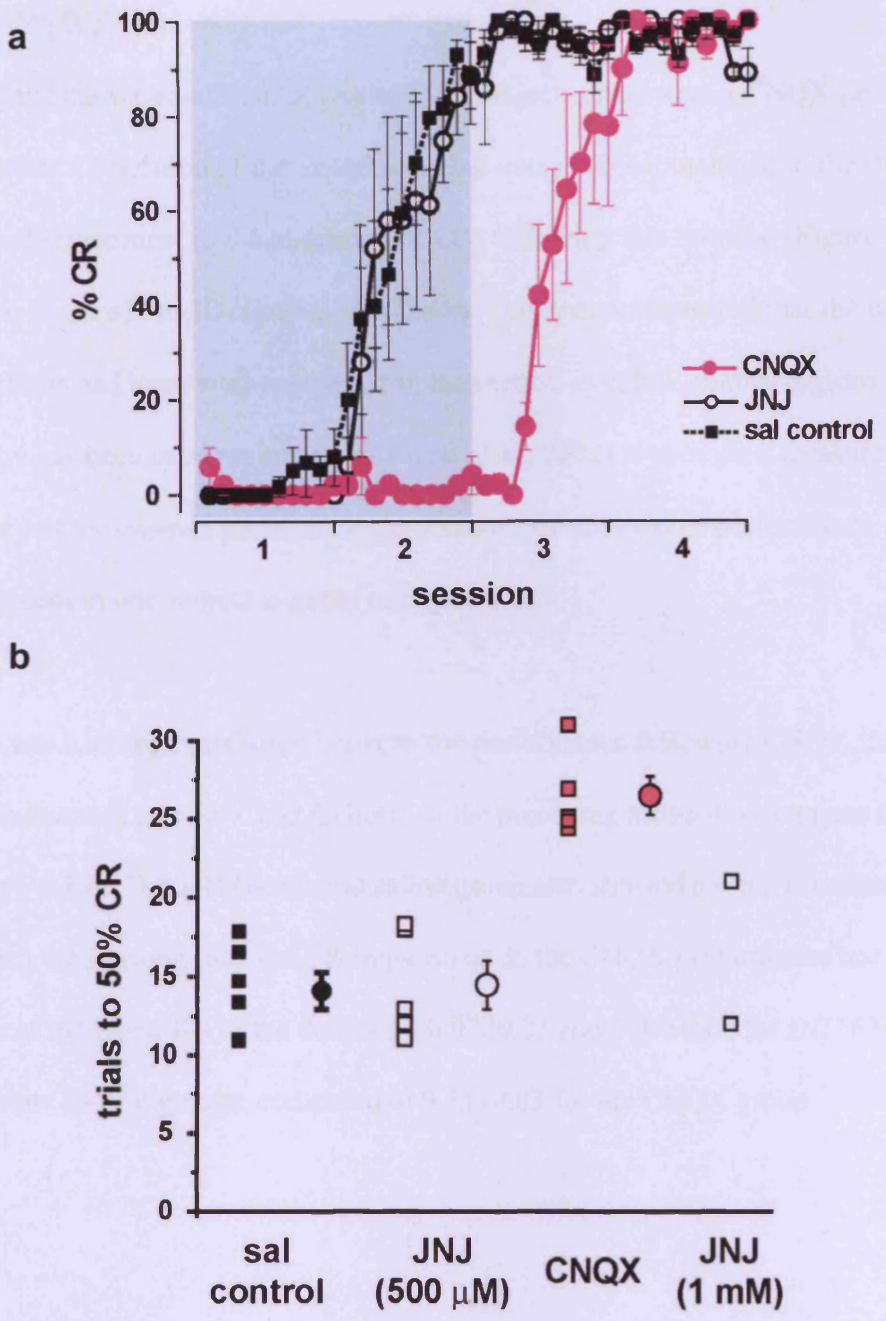


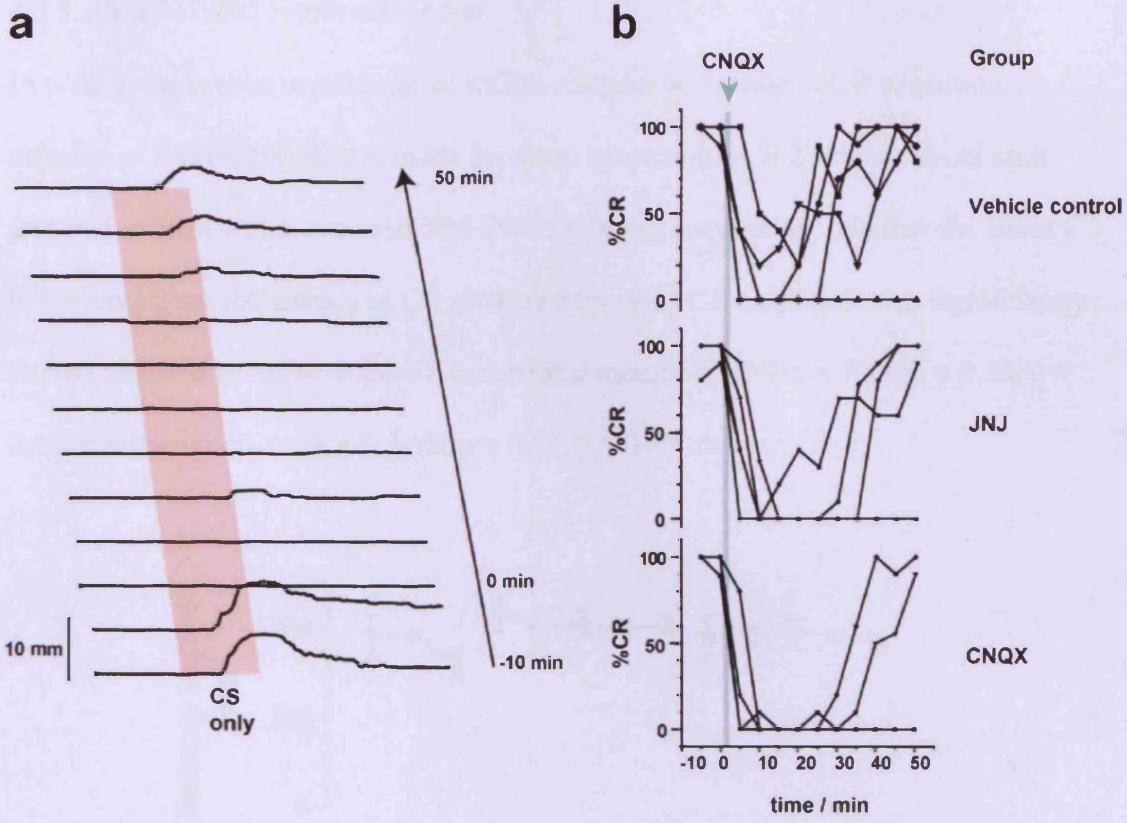
Figure 4.3 Acquisition of conditioning. (a) Acquisition after cortical infusions of JNJ, CNQX or vehicle. The frequency of conditioned responses is expressed as % of trials per 10 trial block showing conditioned responses. Block-by-block CR frequencies are averaged across all subjects in each group and plotted against blocks (10 per session). The first two sessions were conditioning sessions with infusion (coloured blue). (b) A comparison of learning rates. Number of blocks taken to reach 50% CR level by each subject is plotted (left column for each group) along with the group averages (with standard error; n = 5). No group average is available for JNJ (1 mM) group because the group size is too small.

4.3.2. CNQX performance test

Following the six conditioning sessions, all subjects underwent a CNQX performance test. It was a condition of the experiment that every subject included in the study revealed impairment in the execution of CRs following this infusion (Figure 4.4b) with an onset within 10 minutes of infusion. This criterion ensured that the cannula placements had been well-positioned in the cortical eyeblink control regions. This strategy has been used previously (Attwell *et al.*, 2002) to provide a measure of the efficacy of the cannula positioning. An example of this type of performance impairment in one subject is given in Figure 4.4a.

There was a strong correlation between the performance following CNQX infusion and the learning rate for CNQX Group in the preceding acquisition sessions (Figure 4.4c; $r = 0.89$). The JNJ Group and saline group also showed a weak relationship between the learning rate and CR impairment in the CNQX performance test. The slopes of the linear fits to the data were 0.07 ± 0.03 and 0.07 ± 0.04 for JNJ16259685 and saline control groups, compared to 0.15 ± 0.03 for the CNQX group.

Figure 4.4 CNQX performance test. (a) Example from one subject; each trace shows a record of NM displacement during a CS-only trial (every 10^{th} trial in a session). The pink rectangle represents the CS (410 ms) presentation period. The flow of time (also equivalent to trials) is represented by the arrow. The CNQX infusion was made at 0 min for 2 min. (b) Results of CNQX performance test for all subjects ($n = 5$ for each group). (c) Relationship between the learning rates and the impairment in CR% during the subsequent CNQX performance test. Each point represents the number of trials taken by each subject to reach 50% CR level, plotted against the number of trials without CRs during the CNQX performance test (100 trials). All subjects, including those with off-target cannula placements are included.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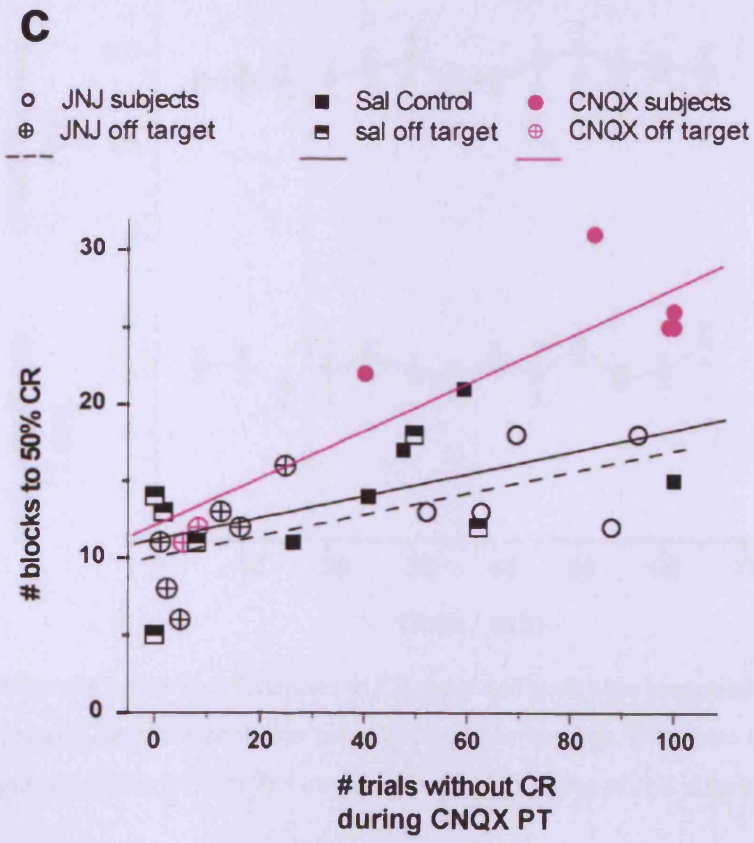
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JNJ subjects
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4.3.3. JNJ16259685 performance test

In order to assess the importance of $mGlu_1$ receptor activation in CR execution, an infusion of JNJ16259685 was made for some subjects (n = 7; 2 subjects from each group; 1 subject which received YM-298198 during acquisition). Neither the latency to CR onset, nor the latency to CR peak, nor the peak CR amplitude was significantly altered after the infusion (Figure 4.5; repeated measures ANOVA F ratio = 0.25, p = 0.62; F ratio = 0.20, p = 0.66; F ratio = 0.03, p = 0.87, respectively).

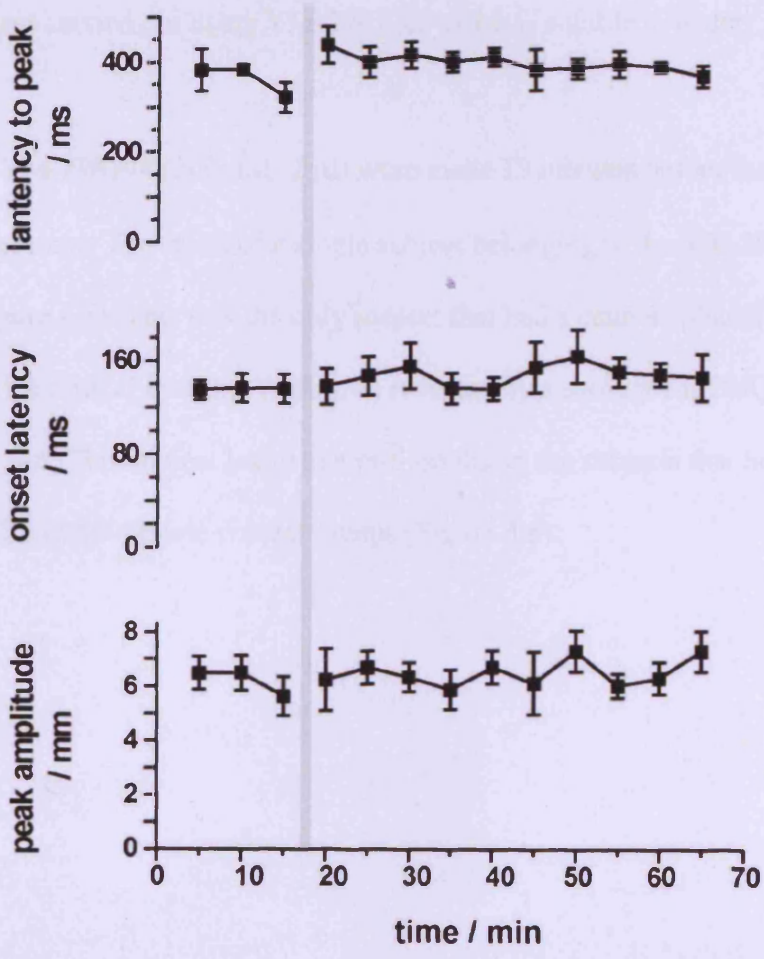


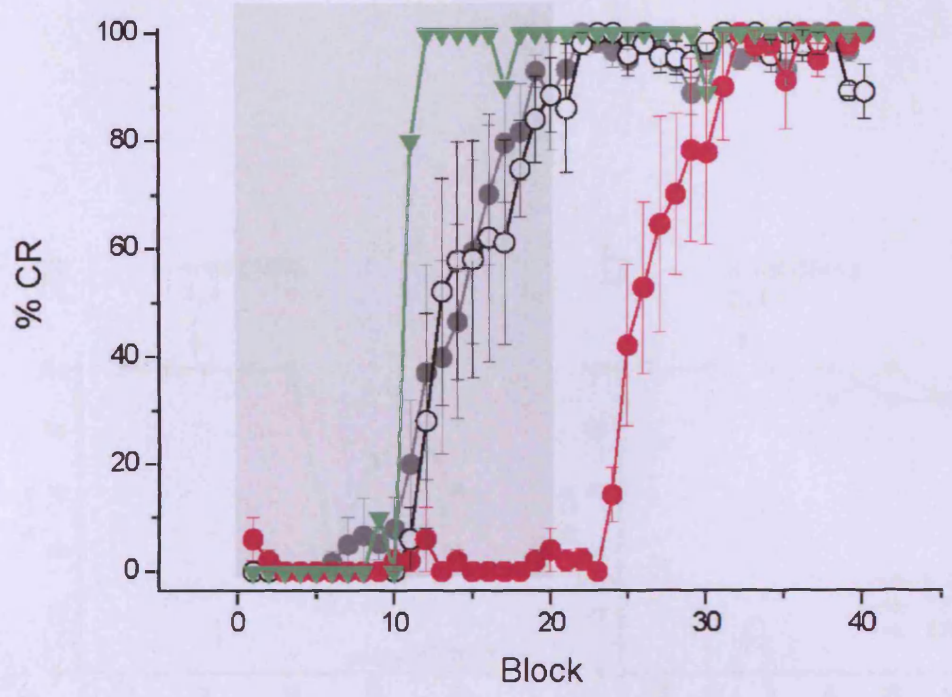
Figure 4.5. JNJ performance test. Latencies to CR onset and peak were measured from the CS onset, and amplitude of CR peak was measured from the baseline. Data from CS-only trials (mean and standard error; n = 7). Grey bar represents the time of JNJ infusion (10 μM, 2 μl).

4.3.4 The effect of YM-298198 on the acquisition of CRs

One limitation with using JNJ16259685 to test $mGlu_1$ receptor functions *in vivo* is that of potential restriction in the drug spread due to lipophilicity. The distribution of JNJ16259685 in the cerebellar cortex may be severely reduced because JNJ16259685 may be distributed preferentially in the cell membranes and other structures with high lipid content which lie close the cannula tip, rather than the extracellular space. A lack of JNJ16259685 effect on the acquisition of CRs may therefore be due to insufficient spread of JNJ16259685 to the relevant eyeblink region. To test this, an acquisition experiment was carried out using YM-298198, which is soluble in water.

Infusions of YM-298198 (200 μM; 2 μl) were made 15 minutes before the start of acquisition sessions. The result of a single subject belonging to the YM-298198 group is shown (Figure 4.6). This was the only subject that had a cannula placed in proximity to the critical eyeblink region, as revealed by a subsequent CNQX performance test. This subject learnt at a rate similar to the subjects that belong to the JNJ16259685 and the vehicle control groups (Figure 4.6).

Figure 4.6. CR acquisition in the presence of YM-298198. The learning curve of a single subject that received YM-298198 (200 μM; green) is superimposed on the learning curves for the JNJ16259685 (open), vehicle control (grey) and CNQX groups (pink).



4.3.5. Effect of an $mGlu_1$ agonist, DHPG, on the execution of CRs

DHPG, a specific $mGlu_1$ agonist, increases the firing rate of Purkinje cells *in vitro* and has been shown to increase baseline firing rate of Purkinje cells *in vivo* (Lingenhoehl *et al.,* 1993). It is therefore likely that DHPG will affect baseline firing rates and possibly CS/US coding in the cerebellar cortex. Therefore, attempts were made to obtain DHPG-induced effects on conditioned responses. DHPG effects on CR topography would indicate that the previous JNJ16259685 infusions through the same cannula would have been to appropriate locations. All subjects included in the study had shown substantial impairment in CR execution following CNQX infusion. In one subject, a 2 µl infusion of 1 mM DHPG (subject T2032, Figure 4.7a) did not affect the CR, whereas a 4 µl infusion led to a partial impairment in the CR execution. To assess whether this partial impairment was due to a general disturbance of the infusion (a volume effect), an infusion of a smaller volume but more concentrated DHPG solution was made (2 µl, 3 mM). This treatment did not produce a reliable impairment in the CR execution (Figure 4.7b).

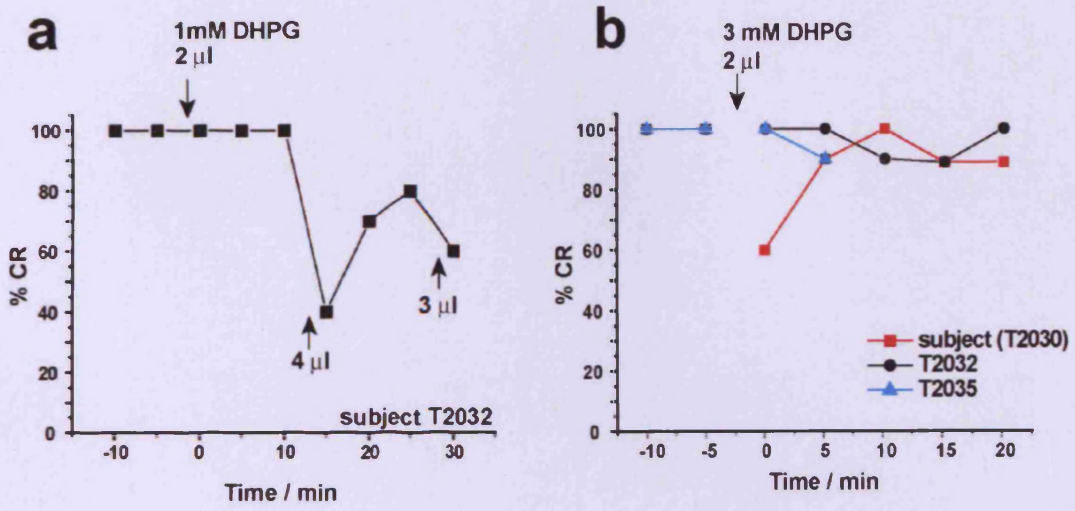


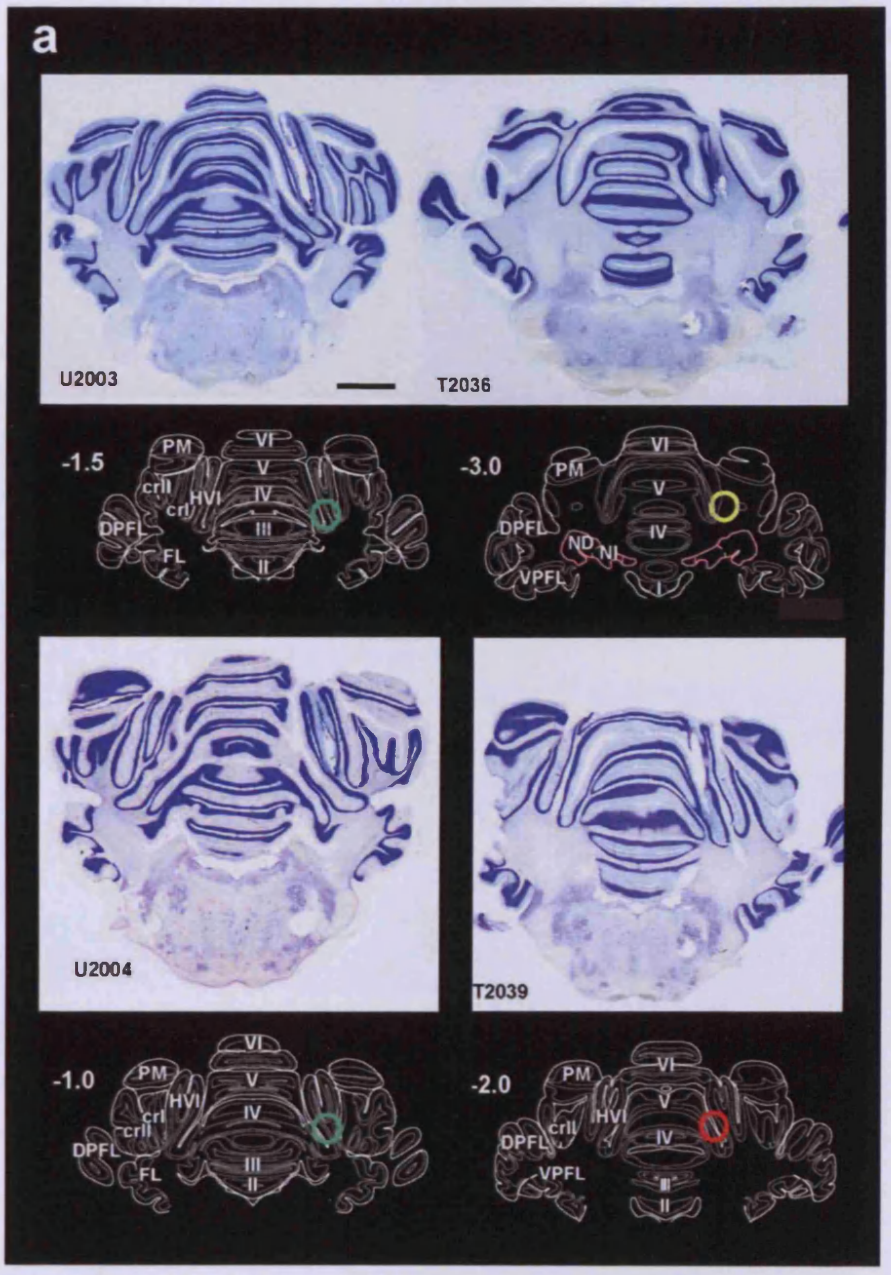
Figure 4.7. DHPG performance test. (a) Various volumes of 1 mM DHPG were infused as indicated with arrows. CR frequency per block (10 trials) for subject T2032. (b) Effect of DHPG (3 mM) infusions in 3 subjects

4.3.5. Post-mortem histology

The cerebella of all subjects were fixed post-mortem in gelatin and sectioned at 50 µm. Locations of the cannula tips were reconstructed from the tracks left in the sections. Examples of Nissl stained sections showing the tracks are shown in Figure 4.8. The location of the injector tip was identified as the lowest point of any cannula track. Typically, the guide cannula produced a broad track of damage and the injector track is seen as a small diameter region of damage projecting about 1 mm below the guide cannula damage. For those subjects that showed major impairment during the CNQX performance test, the tip was located in the tissue in the bottom third of lobule

HVI. The extent of impairment during CNQX performance test is indicated by the colour of the rings marking the locations of cannula tips (red = no effect, yellow = partial effect, green = major; Figures 4.8 and 4.9).

Figure 4.8. Reconstruction of cannula locations. (a) Nissl stained sections with tracks left by cannula guides and injector tips. The bottom illustration represents the location of each cannula tip marked by a ring, interpreted from the sections. The colour of the ring indicates the effectiveness of CNQX in blocking CRs during the performance test. Green = substantial impairment (at least 10 continuous trials without CRs); yellow = partial effect and red = no effect. Value in each panel indicates distance (mm) caudal to lambda. (b) CR frequencies through the CNQX performance test. Scale bar = 2 mm.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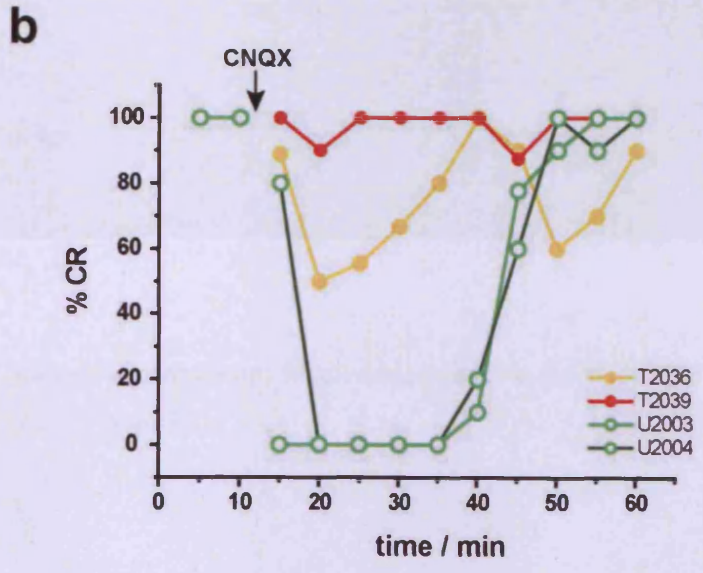
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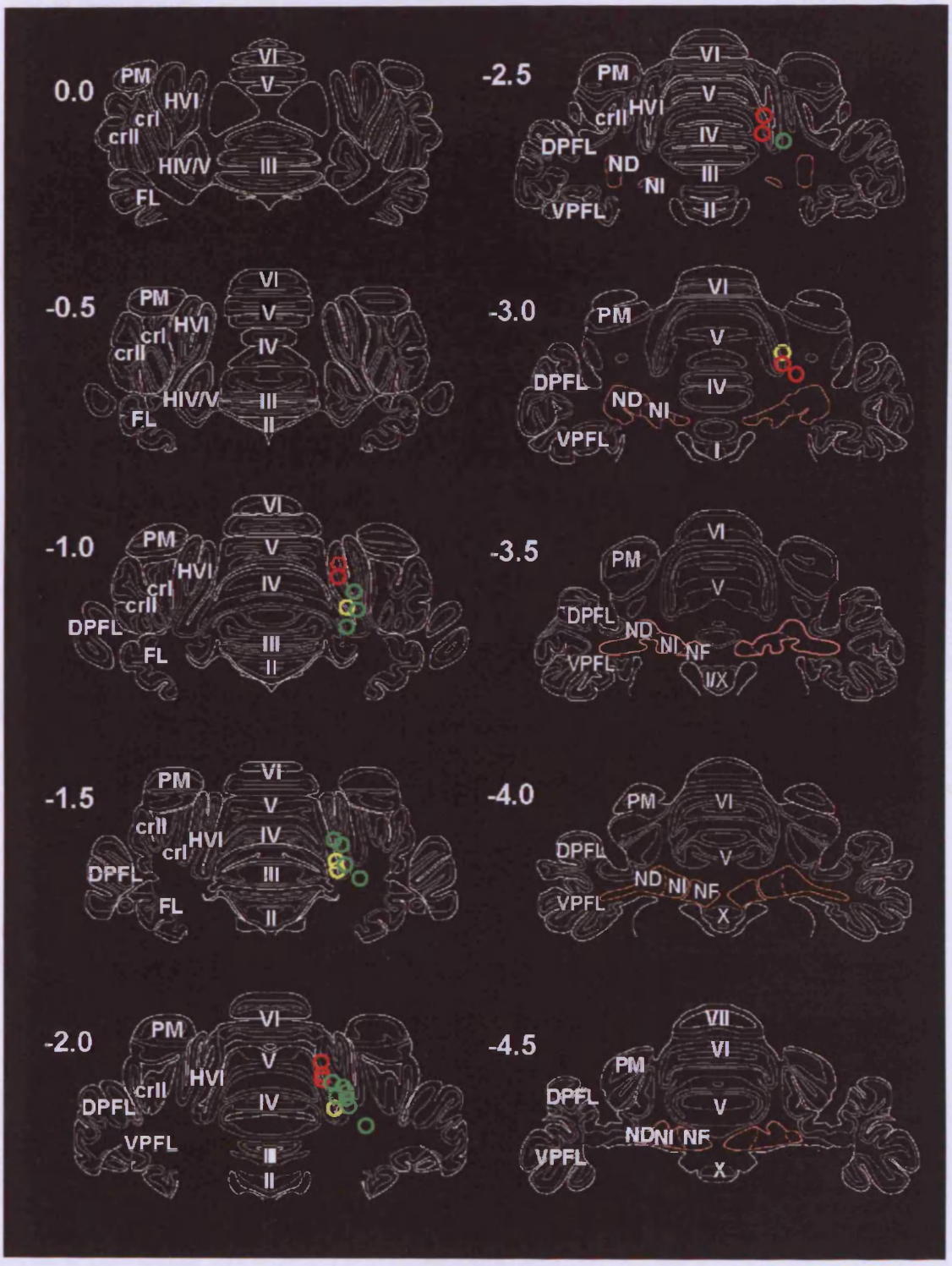

Figure 4.9. Locations of cannula tips for all subjects used in the study. The colour code is the same as that used in Figure 4.7.

4.4. Discussion

Infusion of the non-competitive $mGlu_1$ antagonist JNJ16259685 produced impairments neither in the acquisition nor in the execution of conditioned NM response in this design. If it can be established that the JNJ16259685 reached sufficient concentration to block $mGlu_1$ receptor mechanisms in the relevant cerebellar cortical regions, then this result suggests that conventional, $mGlu_1$ receptor-dependent pf-PC LTD might not underlie at least this form of cerebellum-dependent associative learning.

4.4.1. Was $mGlu_1$ receptor function blocked?

For each subject in the study, assessment of the cannula position, and thus an estimate of the infusion location and drug spread, was obtained from the CNQX performance test. This test determined the extent of impairment in CR execution following the CNQX infusion. All above-criterion subjects showed a substantial impairment of CRs, with a relatively fast onset (within 15 min). However, the extent and time-course of drug spread are likely to be different between CNQX and JNJ16259685. For example, unlike the disodium salt of CNQX, JNJ16259685 is insoluble in water. Additionally, whereas CNQX could produce CR impairments by its action in the granule cell layer (or anywhere that disrupts the transmission of CS-related signals), our hypothesis concerning the role of $mGlu_1$ receptor in learning is restricted to considering its action at the parallel fibre-Purkinje cell synapse. Subjects selected on the basis of the sensitivity of their CRs to cortical CNQX would have included those with either molecular layer or granule cell layer placements. Thus, had JNJ16259685 been effective in blocking learning through its action at the pf-PC synapse, then a bimodal distribution of JNJ16259685 effects on learning might have been predicted: those

with molecular layer placements would have been learning impaired and those with granule cell layer placements might be expected to have normal learning. There was no evidence for any learning impairment in any JNJ16259685 subject and it is unlikely that the CNQX infusions would have affected only the granule cell, and never the molecular layer. Thus the absence of any bimodal distribution of JNJ16259685 learning effects suggests that the learning may be independent of $mGlu_1$ receptor function.

The reason for expressing caution in the interpretation of the absence of effects of JNJ16259685 on learning is that it is unknown whether the drug was delivered at sufficient concentration. To maximise the chances that the dose was sufficient, the concentration of JNJ16259685 used (10 μM) for the main group was 500 times the IC_{50} for blocking $mGlu_1$-EPSP *in vitro*. At higher concentrations than this there are possible interactions with $mGlu_5$, (IC_{50} for $mGlu_5$ is 1.31 μM; (Lavreysen *et al.*, 2004)). Although effects on $mGlu_5$ would be undesirable in this study, it should be noted that a group of two subjects were tested with considerably higher concentrations of JNJ16259685 (1 mM) and still there was no evidence of a learning impairment, despite the possibility that both $mGlu_1$ and $mGlu_5$ receptor functions could have been disturbed.

Although very high doses of JNJ16259685 were used, another factor which may have limited the concentration of JNJ16259685 at the critical site is the lipophilicity of the antagonist. As is suggested by its chemical structure, as well as the need for DMSO to assist its dispersal in aqueous solution, JNJ16259685 is likely to preferentially diffuse into lipids. Since this property may lower the availability of drug in the extracellular

medium, it may have slowed the spread of JNJ16259685. The time allowed for antagonist to diffuse before the conditioning session began i.e. 15 minutes, might have been too short for JNJ concentration to reach an effective concentration at the relevant receptors. However, a preliminary experiment, where a JNJ16259685 (1 mM) infusion was made 1 hour prior to the start of each acquisition session, showed a similar result (n = 2; Figure 4.3).

4.4.2. Behavioural assessment of JNJ16259685 effects

It was difficult to assess, with our behavioural measures, what the optimal infusion protocol should be. This is because JNJ16259685 was found to be behaviourally "neutral" in that it did not block or change the waveform of conditioned responses. Attempts were made to obtain some measure of JNJ16259685 availability in the eyeblink region. JNJ16259685 can block agonist (DHPG) effects (see Chapter 3) so it might have been possible to test for the efficacy of JNJ16259685 infusions by analysing the behavioural effects of its interaction with locally applied DHPG. In order to assess such an interaction, it would be necessary that DHPG itself produces some effect on conditioned responses. DHPG is known to increase the firing rate of Purkinje cells, as well as stellate and basket cells (Karakossian & Otis, 2004), so its application could disrupt the cortical network. However, infusion of DHPG did not lead to reliable changes in conditioned responses. This lack of effects may be because modulation of baseline firing rate may be too subtle to block information transfer within the cerebellar cortex. For example, well-timed changes in the Purkinje cell simple spikes necessary to generate the conditioned response may still be produced on a baseline increase in the firing rate. DHPG is also known to act briefly so the relatively long inter-trial interval used in the experiment (30 seconds) might not be suitable for capturing short-lasting effects produced by DHPG. Another possibility is

that the high concentration of agonist might have caused desensitisation of $mGlu_1$ receptors (Doherty *et al.*, 1999) leading to unreliable effects from repeated infusions. DHPG leaking from the injector prior to the infusion could also cause such desensitisation. To date, this approach has not enabled a satisfactory behavioural assessment of the JNJ16259685 dose.

4.4.3. Alternative cellular mechanisms for associative learning in the cerebellum

Even though it has not yet been possible to establish that the JNJ16259685 infusions produced complete block of $mGlu_1$ receptor function at the critical site during acquisition, the very high concentrations used and the obvious proximity of the infusion sites to the critical zone, as revealed by the CNQX tests, indicates that there may be no requirement for cortical $mGlu_1$ receptor function in the acquisition of NM conditioning. It is known that pf-PC LTD can be induced by a variety of protocols, and not all forms may depend upon $mGlu_1$ receptor activation. This could lead us to the conclusion that a reduction of parallel fibre to Purkinje cell synaptic efficacy may still be an important candidate mechanism in behavioural learning, but pf-PC LTD, considered as a single mechanism as revealed by *in vitro* work, may not be its unique model. There may still be the cellular mechanism for associating CS- and US-related signals at Purkinje cells, but the induction may not require $mGlu_1$ receptor activation. Alternatively, there may be parallel pathways for establishing the association – for example, learning may also require plasticities at other neurons, especially those at molecular layer GABAergic interneurons. These ideas will be discussed in further detail in the final chapter.

General discussion

The experimental work presented in this thesis has assessed the specificity and potency of some $mGlu_1$ antagonists in their action on the cerebellar cortical Purkinje cell and has used one of these compounds to test the effects of blocking $mGlu_1$ receptoraction during the acquisition of a cerebellum-dependent learning task. Although the *in vitro* work revealed that one compound, JNJ16259685, has very high potency and good selectivity, it produced no measurable effects upon the acquisition or execution of NM conditioned responses when delivered to the cerebellar cortical eyeblink control region. In discussing these findings we first consider here the possibility that pf-PC underlies cerebellum-dependent learning but that the molecular model of this plasticity may not be correct. Secondly, we consider the possibility of alternative cellular memory storage mechanisms in the cerebellum.

5.1. Protocol-dependency of pf-PC LTD

The findings described in Chapter 4 do not exclude the possibility that learning in the cerebellum is stored as long-term depression of pf-PC synapses if the molecular model of plasticity that has been characterised *in vitro* is not of the correct form and identical to plasticity seen *in vivo*. Indeed, there is already an indication that pf-PC LTD can be induced by variety of protocols. The form of pf-PC LTD tested in this project was a "conventional" pf-PC LTD, which is induced by repeated conjunctive activations of pf and cf at 1 Hz (Ito, 2001). As discussed in Chapter 1, this form of pf-PC LTD is associative and dependent on $mGlu_1$ receptor activation. Further investigations revealed, however, that some protocols induce pf-PCLTD that does not

require this exact condition for the induction. For example one form of pf-PC LTD can be induced by pf stimulation alone (Hartell, 1996; Schreurs & Alkon, 1993).

The stimulus dependency of pf-PC LTD induction is reminiscent of the stimulus dependency involved in evoking a "supralinear" rise in Ca^{2+} concentration in Purkinje cell dendritic spines. It can be evoked by pairing a burst of weak activation of parallel fibres, followed by climbing fibre activation with a suitable ISI (~100 ms in Wang *et al.*, 2000; ~80 ms in Safo & Regehr, 2008). This rise in the intracellular Ca^{2+} concentration is more than the sum of Ca^{2+} concentration rises produced by the pf and cf separately and it is dependent on $mGlu_1$ receptor activation. However, when the pf stimulus strength is increased, the process becomes $mGlu_1$-independent and eventually non-associative i.e. independent of climbing fibre activation. Similar protocol dependency has been observed for endocannabinoid production (Brenowitz & Regehr, 2005), another molecule that is known to be involved in the pf-PC LTD induction.

If cerebellum dependent learning can be supported by an $mGlu_1$ receptor-independent form of pf-PC LTD, this would be consistent with the negative result reported in Chapter 4.

5.2. Physiological patterns of parallel fibre activation

That there is more than one molecular mechanism of pf-PC LTD induction leads to questioning which, if any, is the physiologically relevant induction mechanism. Because the differences in the molecular mechanisms described above are due to differences in the parallel fibre stimulation strength used in the induction protocols, an

important question arises: what is a physiologically relevant pattern of parallel fibre inputs? To fully answer this question, a detailed knowledge of the incoming parallel fibre activities is required. The recent studies of granule cell firing pattern *in vivo*, where granule cells responded to peripheral stimulations with high frequency bursts (Chadderton *et al.*, 2004; Jorntell & Ekerot, 2006; Hensbroek et al., 2006) have already been mentioned in Chapter 1. How such patterns of granule cell activity translate into postsynaptic responses in Purkinje cell dendrites is unknown. Factors that influence such responses include the density of activated parallel fibres, the number of granule cells contributing to the pattern of activity, efficacy of parallel fibre-Purkinje cell synapses and, finally, how the EPSPs and the feed-forward inhibitory inputs integrate in Purkinje cell dendrites. Such questions are subjects for future investigation.

5.3. Physiological pattern of cf activation

A further problem in matching mechanisms defined *in vitro* with those underlying learning *in vivo* is the behaviour of the climbing fibre inputs. Climbing fibre stimulations implemented in the slice investigations do not necessarily mimic behaviourally relevant climbing fibre activity. Classically, the climbing fibre stimulation used in inducing pf-LTD in slices uses 1 Hz stimulation, because complex spikes *in vivo* are reported to occur, on average, at this rate (Armstrong *et al.*, 1968). There is, however, an old and neglected finding that the climbing fibres fire in bursts (Eccles *et al.*, 1966; Armstrong & Rawson, 1979).

In discussing the behaviour of cf inputs, it is important to remember that each complex spike does not necessarily follow just one event or action potential in the

climbing fibre. The consequences of compound climbing fibre events can be observed in non-spiking Purkinje cells, such as those damaged by impaling with a sharp electrode (Eccles *et al.*, 1966). Such recordings reveal the presence of large EPSPs, believed to originate from climbing fibre activities. An analysis of climbing fibre EPSPs indicates that in awake, behaving animals, climbing fibres fire in bursts (Armstrong & Rawson, 1979). The presence of bursts of large EPSPs, which show paired-pulse depression, was recently confirmed in floccular Purkinje cells from awake rabbits. It was shown that these cells received 1-5 EPSPs at approximately 500 Hz. The greater the number of EPSPs within each compound climbing fibre event, the less frequently it occurred. However, during vestibular stimulation, when the inferior olivary neurons projecting to the flocculus can be driven by image motion in the retina, there is an increased occurrence of compound climbing fibre events with greater number of EPSPs. Thus it was suggested that meaningful information can be coded in by the number of spikes in the cf burst and/or by the interburst intervals (Maruta *et al.*, 2007).

5.4. How might the climbing fibre burst influence pf-PC LTD induction mechanisms?

It is thought that the major role of the climbing fibre in the induction of pf-PC LTD is to raise the intracellular Ca^{2+} concentration in the Purkinje cell. Because the cf-PC synapses are powerful, a single climbing fibre EPSP leads to a substantial rise in the intracellular Ca^{2+} concentration via activation of voltage-gated calcium channels. Since this Ca^{2+} concentration rise to a single EPSP is already quite large, it is questionable whether the burst climbing fibre input adds significantly to the elevation of the intracellular Ca^{2+} concentration via voltage gated calcium channels. However,

as reports on the supralinear Ca^{2+} rise suggest (Wang *et al.*, 2000), it is possible for the intracellular Ca^{2+} concentration to rise higher than the levels achieved during a climbing fibre response alone. It is possible that a greater Ca^{2+} rise may be achieved, for example, by additional Ca^{2+} release from internal stores.

Climbing fibres also release corticotrophin release factor, CRF (Palkovits *et al.*, 1987), a peptide that reduces the after hyperpolarization (AHP) in Purkinje cells (Fox & Gruol, 1993). CRF is required for pf-LTD induction (Miyata *et al.*, 1999b), as well as for climbing fibre-LTD induction (Schmolesky *et al.*, 2007;Barmack & Young, III, 1990). It has also been suggested that CRF is released from climbing fibres in an activity-dependent manner (Miyata *et al.*, 1999), and so this may be another aspect of climbing fibre function that may be enhanced during high frequency activity. Although the expression level of CRF mRNA seems to depend upon increases in inferior olive activity in response to vestibular stimulation (Barmack & Young, III, 1990), it is unknown whether cf burst activity leads to increased release of CRF.

5.5. Multiple induction mechanisms *in vivo*?

It is possible that several different forms of pf-PC LTD coexist *in vivo*. One possibility is that pf-PC LTD induced *in vivo* has different molecular mechanisms depending on the circumstances. This suggestion stems from the parameter (mainly frequency) dependence of VOR gain modifications observed in mice that lack CamKIV (Boyden *et al.*, 2004). In these mice, the late phase of pf-PC LTD induction is impaired, so that the depression that is induced at the pf-PC synapses cannot be maintained. These mice showed a deficit in the maintenance of VOR gain increase when observed 24 hours after the training, specifically at frequencies including and

higher than 1 Hz. At lower frequencies, e.g. 0.5 Hz, there was no difference in the abilities of the wild type and the mutant mice to maintain VOR gain increases. The authors suggested that VOR gain modifications at low frequencies occur independently of CamKIV activation. However, in these experiments, the changes in the VOR gain induced by low stimulation frequencies e.g. 0.5 Hz were much smaller. Therefore, it is not clear whether the gain change induced at such low frequencies was too small to reveal differences between the wild type and the mutant mice. Frequency-dependence of VOR modification has been observed in other species, for example in monkeys. There is a behaviourally observable difference between the gain and the time-course of VOR, when different frequencies of vestibular stimulation are used (Raymond & Lisberger, 1996). However, these differences do not necessarily require multiple molecular mechanisms, as the protocol-dependency of the VOR can also be explained in terms of multiple sites of memory formation. In particular there is strong empirical and theoretical evidence for different extents of memory storages at cerebellar cortical and brainstem sites depending on the velocity of vestibular stimuli (Porrill & Dean, 2007). Therefore, it may be premature to conclude that there are multiple molecular mechanisms of memory storage in the cerebellar cortex. To test this conjecture and whether there is a protocol that induces an $mGlu_1$ receptor-dependent form of NM response conditioning, systematic variations in the CS and US presentation in the presence of an $mGlu_1$ antagonist in HVI may be required.

5.6. Pauses in PC simple spikes as the model of learned output

How does the firing pattern of relevant Purkinje cells change during conditioning, and does the pf-PC LTD satisfactorily explain the change? Such studies are scarce because of the natural difficulty in maintaining a stable recording from identified

Purkinje cells in awake animals. However, some PC recording data are available from a study by Berthier and Moore (Berthier & Moore, 1986). Recordings were made from Purkinje cells in the HVI during CS and US presentations. The subjects were trained to discriminate two different tone frequencies. One CS was reinforced and the other was not (the CS+ and CS-, respectively). After discrimination training, the subjects blinked to the CS+ with high probability and with much lower probability to the CS-. Thus on some trials, they do not respond to the CS+ and they produced CRs erroneously on CS- trials. This allowed the authors to record activities related to CRs, rather than the CS. The response types were various. Some cells increased firing that correlated with CR, but some showed an inhibitory response that preceded the CR. However, most of the cells recorded did not have a climbing fibre response that related to the US. One cell out of about seventy cells responded to the US consistently with a complex spike, and its "pre-CR" responses were simple spike pauses. All cells that increased simple spikes preceding CR did NOT show a complex spike after US.

In a more recent study, recordings were made from identified PCs in the C3 zone in lobule HVI of decerebrate ferrets. After paired presentations of CS (mossy fibre or forelimb stimulations) with US, Purkinje cells acquired a CR-like firing pattern following conditioning (Jirenhed *et al.*, 2007). That is, there was a pause in the Purkinje cell simple spikes in response to the CS, preceding and overlapping the time of the US arrival. When mossy fibre stimulation was presented without the US, the learned pause gradually was replaced by continued generation of simple spikes, reminiscent of the behaviourally observed extinction. In this model of learning, learning manifests as well-timed pauses in PC simple spikes. No behavioural output was available in these studies, as the subjects were curarized in order to maintain

recording stability. The simple spike pause appears very similar in its profile to the overt CRs that have been reported previously in the non-curarized state of this decerebrate preparation (Hesslow & Ivarsson, 1994), and it is likely that the PC simple spike pause is highly correlated with the CR and may, in future work, be demonstrated to be causal.

5.7. Is the pf-PC LTD alone satisfactory in explaining learnt pauses in Purkinje cell spikes?

Though learning such as NM/eyeblink conditioning, might depend upon pf-PC LTD it might additionally or alternatively depend upon changes in molecular layer GABAergic interneurons. At least in the slice, Purkinje cells are spontaneously active when isolated from synaptic events (Hausser & Clark, 1997). That is, to induce such well-timed pauses in Purkinje cell firing, well-timed inhibitory inputs to the PCs might also be required, and this would require learning-related plasticity at molecular layer interneurons. Two possible sites for such plasticity are at interneuron-PC synapses, and at pf-interneuron synapses.

5.8. Plasticity at molecular layer interneuron-PC synapses

Two forms of plasticity at interneuron-PC synapses in slices have been described. One has been termed rebound potentiation of IPSCs (Kano *et al.*, 1992), where the sensitivity to GABA in Purkinje cell increases following climbing fibre input. This change is reflected in the increase in amplitude, but not the frequency, of spontaneous IPSCs. Purkinje cell responses to application of GABA also increase. In contrast, when evoked IPSCs are paired with climbing fibre inputs, a long-lasting depression of evoked IPSCs is observed (Mittmann & Hausser, 2007). The difference between the

results in these two experiments, where one produces potentiation and the other produces depression, was explained in terms of different experimental condition (caesium-based internal solution, compared with potassium-based solution in (Mittmann & Hausser, 2007). Another difference between their studies is in the nature of the IPSCs. Mittmann and Hausser studied IPSCs evoked by electrically stimulating the molecular layer, whereas Kano et al studied spontaneous IPSCs. Neither of the studies would satisfactorily explain well-timed pauses in PCs simple spike activity. For example, the consequence of the long-term depression of evoked IPSCs, together with long-term depression of pf-PC LTD, was to shorten brief pauses in Purkinje cell simple spikes immediately following parallel fibre stimulations in the molecular layer (Steuber *et al.*, 2007). A fuller answer to these questions will require additional analyses that extend the range of experimental protocols to include, for example, burst input from the molecular layer interneurons.

5.9. Plasticity at parallel fibre-interneuron synapses

An important alternative mechanism for cerebellum-dependent learning plasticity concerns changes at the pf-interneuron synapses. If, as evidence suggests, the learning is dependent upon climbing fibre instructions, then this suggestion implies that the molecular layer interneurons also receive climbing fibre signals, either directly or indirectly. There are at least two ways in which signals from the climbing fibres can reach stellate/basket cells via direct and indirect routes. A direct route here refers to a route of transmission whereby transmitter released by climbing fibres is detected by the molecular layer interneurons themselves. An indirect route refers to climbing fibre input that arrives at stellate/basket via an intermediary neuron or neurons. This could occur, for example, via climbing fibre connection to Golgi cells and would be

reflected in the granule cell activity pattern. Anatomically, climbing fibres are known to contact Golgi cell somata and possibly dendrites via collaterals that branch within the cortex (Scheibel & Scheibel, 1954; Sugihara *et al.*, 1999). However, it is not known to what extent climbing fibre input influences Golgi cell activity. Furthermore, if climbing fibre activities evoke a particular pattern of activity of Golgi cells, how do such activities translate into granule cell activity? Simultaneous Golgi and granule cell recordings *in vivo* are not available, but separate recordings of Golgi and granule cell activities from the same region, i.e. underlying the same Purkinje cell microzone are available (Jorntell and Ekerot, 2006). The assumption is that the Golgi cells, because their axons ramify underneath their cell bodies, contact the local granule cells. Golgi cells in the forelimb area of C3 zone respond to cutaneous stimulations of the forelimb with brief bursts. Granule cells from the same area receive slow, rather than fast, IPSPs (Jorntell and Ekerot, 2006) with small amplitudes. This is consistent with a suggestion that a substantial proportion of GABAergic inhibition of granule cells is TTX-insensitive, tonic inhibition (Brickley et al., 1996). Thus climbing fibre activity may affect the Golgi cell activity but currently the importance of phasic inhibition on granule cell activities is unknown.

Is the suggestion of a direct climbing fibre input to molecular layer interneurons credible? There is direct, anatomical evidence for the sparse connections made by climbing fibre synapses on molecular layer interneurons (Hamori & Szentagothai, 1980; Sugihara *et al.*, 1999). Despite this suggestion that the connection may be very weak, intracellular recordings from interneurons show a slow, but significant EPSP following olivary or climbing fibre stimulation (Jorntell & Ekerot, 2003; Szapiro & Barbour, 2007), confirming an earlier report of the presence of a weak activation of

these neurons by climbing fibres (Eccles et al., 66). This slow transmission from the climbing fibres to the molecular layer interneurons has been suggested to occur via spillover of glutamate from neighbouring climbing fibre-Purkinje cell terminals (Szapiro & Barbour, 2007). The effects can be detected as a slow AMPA- and NMDA receptor-mediated current, whose all-or-none characteristics followed those of the climbing fibre responses in neighbouring Purkinje cells. Clearly, burst firing in climbing fibre inputs would give increased spillover that could increase the security of climbing fibre influence upon the interneurons, the magnitude of the effect and potentially the size of the population upon which the spillover effects take place. These spillover effects upon cortical interneurons could even underlie the post complex spike pause (Simpson *et al.*, 1996; Szapiro & Barbour, 2007).

Even with functional climbing fibre inputs to molecular layer interneurons there is still uncertainty as to whether or not they can affect the efficacy of parallel fibre-interneuron synapses. Furthermore it will be important to understand possible differences in the information represented by the interneurons and the Purkinje cells to which they project. In other words, is there a difference in the populations of granule cells pooled by PCs and interneurons and is it significant? This question arises because of the tangential distribution of stellate and basket cell axons (Figure 1.2, Chapter 1). This arrangement may have the effect of increasing the sampling area of a given Purkinje cell. The nature of the climbing fibre signals to the interneurons influencing Purkinje cells is suggested to reflect that of the local climbing fibre (Jorntell and Ekerot, 2003). However, given the climbing fibre inputs to stellate and basket cells are not exclusive, unlike the arrangement found for PCs, and for stellate and basket cells to influence the PC activity in a manner specific to a microzone, it

may be expected that the arrangements of stellate/basket cell dendrites conform to the climbing fibre innervation patterns. These are important topics for future investigations.

5.10. Plasticity at molecular layer interneurons and pauses in Purkinje cell firing

Marr (1969) postulated that feedforward inhibition to the PC provides a normalising function, as well as an efficient way of preventing postsynaptic spiking, because the inhibition follows shortly after parallel fibre input to PC. He suggested that a simple sum of the inhibitory and excitatory inputs determines the output of Purkinje cells. Given this "balancing" feed-forward inhibitory input, the result of a parallel fibre depression should be a net inhibition of those Purkinje cells driven by those parallel fibres, without any requirement for plasticity at GABAergic synapses. Although Albus (1971) pointed out, that plasticity of the inhibitory inputs to Purkinje cell would give greater memory storage capacity, he did not suggest this mechanism for producing pauses in PC activity.

Information on the proportion of excitation and inhibition induced following parallel fibre activation in Purkinje cells is scarce. Some parallel fibre activation is likely to lead solely to inhibition in Purkinje cells, because axons of stellate/basket cells course laterally to produce so called "off-beam" inhibition. Different proportions of excitation and inhibition and their read out as measured by how they alter the firing pattern of Purkinje cells, have been investigated in the slice (Mittmann and Hausser, 2007). Here, inhibitory and excitatory inputs were generated in PC somata using a dynamic clamp, and the ratios between EPSC and IPSC were varied. As expected, there was a clear indication that the spiking pattern, and especially the length of pause

following a EPSC/IPSC "cascade", is influenced by the proportion of IPSCs present, i.e. larger the IPSC, the longer the pause. However, the proportion of EPSC/IPSC following physiological parallel fibre activity pattern is still unknown. It remains unclear whether pauses in Purkinje cell spikes occur and to what extent they depend upon previous plasticity.

5.11. Conclusion

The results from this project suggest, but did not confirm, that conventional $mGlu_1$ receptor-dependent pf-PC LTD does not underlie cerebellum-dependent associative learning. Further experiments are required to confirm this result. Nonetheless, to determine whether pf-PC LTD underlies cerebellum dependent learning, and if it does, to what extent it is able to explain behavioural learning, may be keys to understanding the mechanisms of cerebellar functions. These investigations also highlight the limitations of *in vitro* investigations as a means to describe the real nature of behaviourally relevant neural signals. At present, they have rarely matched a requirement for determining patterns of incoming synaptic events and the transformation of these into an output of a neuron, as well as how these might be modified during learning. Such analyses are made more difficult because there still remain many unknowns about the physiology of cerebellar cortical neurons. A proper understanding of information processing in the cerebellum requires better descriptions of the physiology of these neuronal types *in vivo* and a better understanding of the integration of these cerebellar functions with extracerebellar brain mechanisms.

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